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Liquid chromatography–electrospray mass spectrometry (LC–MS) method for determination of esmolol concentration in human plasma

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Abstract

Esmolol is a very short-acting β-blocker commonly used in critically ill patients. To study the pharmacokinetics of esmolol in pediatric patients, a liquid chromatography–electrospray mass spectrometry (LC–MS) method to quantitate esmolol concentration in plasma was developed. Following methylene chloride extraction from 200 μl aliquots of plasma containing internal standard and reconstitution in 0.05% formic acid, 10 μl are injected onto column, eluted using a methanol/formic acid gradient over 15 min, and monitored with selected ion recording at 296.2 and 282.2. The assay is linear between 2 and 1000 ng/ml. The intra-day and inter-day coefficients of variation are less than 8 and 10%, respectively.

Keywords: Esmolol

1. Introduction

Esmolol (Brevibloc) is a β_1-selective antagonist with a very short duration of action. Intravenous esmolol is administered when β-blockade of short duration is required, such as in critically ill patients in whom adverse effects of bradycardia, heart failure, or hypotension may necessitate rapid reversal of β-blockade. In adults, esmolol has a terminal half-life of approximately 9 min and an apparent volume of distribution of approximately 2 l/kg. The drug contains an ester linkage (Fig. 1), and is hydrolyzed rapidly by erythrocyte esterases. The elimination half-life of the inactive carboxylic metabolite of esmolol is approximately 4 h [1].

The pharmacokinetics of esmolol in pediatric patients has not been well studied. One consideration when developing a pharmacokinetic study for infants, children and adolescents is the blood volume required for drug quantitation. As previous analytic methods have relied on HPLC with UV detection, we developed a LC–MS method that improved sensitivity and specificity utilizing significantly smaller sample volumes.

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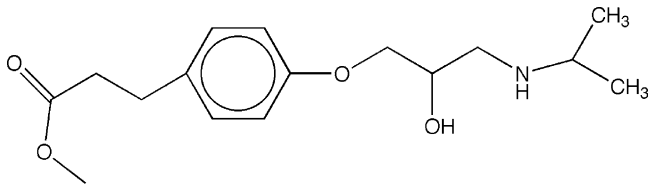

Fig. 1. Chemical structure of esmolol.

Esmolol is inherently unstable in whole blood due to the ubiquitous presence of red cell esterases [2]. Most previous studies of esmolol pharmacokinetics have utilized sodium fluoride (NaF) to inactivate the red cell esterases [3–5]. However, a recent report suggested that NaF in concentrations as high as 30 mg/ml were not effective in stabilizing esmolol in whole blood specimens [6], raising significant concerns about the reliability of previously published results. We therefore studied esmolol stability and the effects of NaF inactivation under the conditions that would be encountered during a multi-site trial of esmolol pharmacokinetics in pediatric patients.

2. Experimental

2.1. Chemicals and reagents

Esmolol HCl (mw = 295.2) reference standard and the internal standard (mw = 281.2) (methyl-3-(4-(2-hydroxy- 3-(ethylamino)-propoxyl)phenyl)propionate, HCl) were obtained from Baxter Healthcare Corporation (New Providence, NJ). Methylene chloride, formic acid (90%), HPLC grade water, HPLC grade methanol and sodium fluoride were purchased from Fisher Scientific Inc. (Pittsburgh, PA).

2.2. Instrument and analytical conditions

The HPLC system (Waters Assoc., Milford, MA), consisted of a Waters 2690 liquid chromatograph system equipped with an autosampler, an automatic electronic degasser, an automatic thermostatic column compartment and the Waters Micromass ZMD mass spectrometer. A 50 mm × 2.1 mm Xterra MSC18 (3 μm) analytical column (Waters Assoc.) and a 4 mm × 2 mm C_{18} guard column (Phenomenex, Torrance, CA) were used. The columns were maintained at 40 °C.

The mobile phase consisted of water with 0.05% formic acid and 1% methanol (mobile phase A) and methanol with 0.05% formic acid (mobile phase B). Mobile phase A was prepared by the addition of 10 ml of HPLC grade methanol and 556 μl of 90% formic acid to 990 ml of HPLC grade water. Mobile phase B was prepared by the addition of 556 μl of 90% formic acid to 1000 ml HPLC grade methanol. A gradient elution at flow rate of 250 μl/min was used by online mixing of HPLC eluents (Table 1) in the following manner: 95% A to 20% A in 5 min, hold at 20% A for 3 min, then a linear gradient to 95% A over 1 min, followed by 6 min of column equilibrium at 95% A, for a total run time of 15 min per sample. Under these conditions, esmolol eluted at approximately 5.7 min and the internal standard at 5.4 min (Fig. 2).

2.3. Mass spectrometric conditions

The Waters Micromass ZMD mass spectrometer was operated using the electrospray ionization (ESI) source. All measurements were carried out using the positive ESI. A source temperature of 110 °C and a desolvation temperature of 300 °C were optimal. Nitrogen was used as a nebulizer gas and a drying gas at flow-rates of 460 ± 5 and 60 ± 3 l/h, respectively. The capillary voltage

Table 1
Chromatographic conditions

Time (min)	Mobile phase A	Mobile phase B	Curve
0	95	5	1
5	20	80	6
8	20	80	11
9	95	5	6
15	95	5	11

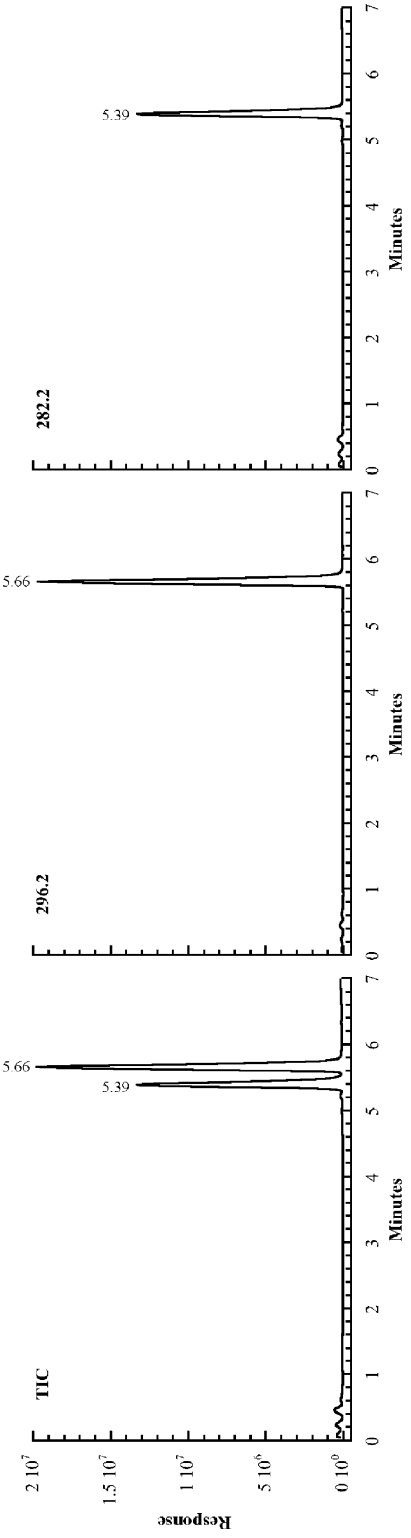

Fig. 2. Chromatogram of a 250 ng/ml injection: total ion channel (TIC), esmolol at 296.2, and internal standard at 282.2.

was 0.76 V and the cone voltage was 36 V. The scan time was 0.4 s, the inter-channel delay was 0.02 s, and the data collection time was 7 min. Selected-ion recording (SIR) was done at 296.2 and 282.2. Detection was performed using the pseudo-molecular ion $[M + H]^+$ of esmolol and the internal standard, respectively.

Peak area ratios were used for calculation and the calibration curve was fitted to a weighted ($1/x$) linear regression model. The Apek Track Peak Integration and Mass Lynx V3.5 data analysis software were used.

2.4. Preparation of plasma standard and quality control samples

Esmolol stock solution (1000 μg/ml) was prepared in water/methanol, 4:1, v/v. Working solutions of esmolol were prepared by serial dilution with H_2O. Quality control (QC) samples were prepared using working solutions prepared from a separate stock solution. Internal standard stock solution (500 μg/ml) was prepared using water/methanol, 4:1 v/v, with further dilutions in water for a working solution (2 μg/ml). Esmolol spiked in plasma (with sodium fluoride 10 mg/ml) was used for esmolol standards. The calibration curve consisted of seven plasma standards, 2, 15, 25, 250, 500, 750 and 1000 ng/ml. QC's were 2, 6, 100, 400 and 800 ng/ml.

2.5. Sample extraction procedures

Esmolol standards were kept at −70 °C. On the day of analysis, samples were thawed at room temperature for 10 min. Standards were centrifuged at 14,000 rpm for 4 min and placed on ice after centrifugation. Plasma (0.2 ml) was transferred into a 13 mm × 100 mm glass culture tube (with a PTEF-faced rubber-lined cap) containing 30 μl of 2 μg/ml internal standard, and vortexed. 3.5 ml of methylene chloride was added to each tube. After 12 min on a tube shaker, the sample was centrifuged at 1900 × g for 10 min. Three milliliters of the clear organic phase was transferred into 12 mm × 75 mm culture tubes, evaporated with nitrogen purging and reconstituted with 400 μl of water with 0.05% formic acid. Ten microliters were injected onto the HPLC column.

2.6. Method validation

Assay precision was determined using five determinations per concentration (2, 125, 500 and 750 μg/ml). The accuracy and the lowest level of quantitation, was determined by replicate analysis of the QC samples. Recovery experiments were performed by comparing the analytical results for extracted samples at two concentrations with unextracted standards. The internal standard was not used for recovery experiments.

2.7. Stability of drug in whole blood

Whole blood was obtained from either The Children's Hospital of Philadelphia blood bank (stored) or from normal volunteers (fresh). For stored whole blood specimens NaF was added to blood to achieve a concentration of 10 mg/ml. Fresh whole blood was drawn directly into grey top tubes (Becton Dickinson 367001) containing 10 mg NaF and K+ oxalate, or into green top tubes containing lithium heparin.

Whole blood aliquots were placed in a 37 °C water bath to simulate body temperature. At time 0, aliquots were spiked with esmolol, to produce a final concentration of approximately 2.6 μg/ml. Specimens were then placed in room temperature or on ice. Aliquots of 3–4 ml of spiked whole blood were centrifuged (1860 × g 5 min) at 4 °C at 3, 10, 20, 30, 60, and 120 min after the addition of esmolol. Plasma was separated, collected and stored at −20 °C for analysis.

2.8. Assay interference by commonly used drugs

Esmolol is often administered to critically ill patients. LC–MS methods are highly specific, but a number of commonly utilized medications were screened to assure assay specificity. Medications studied were a select number of commonly utilized drugs in the pediatric intensive care setting, including the antibiotic cefazolin, the muscle relaxant pancuronium, the opiates fentanyl and morphine, and the benzodiazepine midazolam. For these studies, plasma was spiked with pharmacologic concentrations of cefazolin (100 μg/ml), pancuronium (0.3 μg/ml), fentanyl (15 ng/ml), morphine (150 ng/ml), midazolam (90 ng/ml), and acetaminophen (30 μg/ml), and then analyzed.

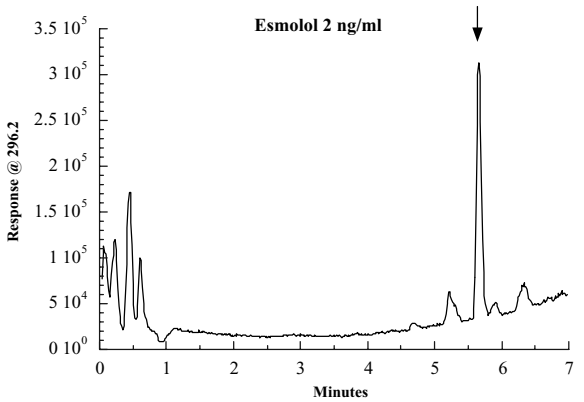

Fig. 3. Chromatogram of esmolol at the LOQ.

3. Results

3.1. Calibration curve

The lower limit of quantitation (LOQ) of esmolol was 2 ng/ml. At this concentration, no interference in blank plasma was detected, and the response was greater than 10 times baseline noise (Fig. 3). A linear regression with weighting to the inverse of concentration was performed. The correlation coefficient for calibration curves was greater than 0.98 in all cases (Fig. 4). At the LOQ, there was less than 15% deviation from the nominal concentration, and, except for a single determination at 500 ng/ml, was less than 10% deviation at all other concentrations (Table 2).

3.2. Method validation

The precision, determined at each concentration level, did not exceed a 10% coefficient of variation (CV) (Table 3). The inter-day CV ranged from 5 to 9% (Table 4). The accuracy of the method was performed using five determinations per concentration for five concentrations in the range of expected concentrations. The mean value was within 10% of the actual value except at LOQ, where it was within 20% (Table 5). Recovery exceeded 85% at 25 and 250 ng/ml concentrations (Table 6).

3.3. Interference from commonly used drugs

Cefazolin, acetaminophen, morphine, fentanyl, midazolam and pancuronium did not co-elute with esmolol.

3.4. Stability of drug in whole blood

At room temperature, esmolol was unstable in fresh whole blood, decaying with an in vitro half-life of approximately 27 min. The addition of NaF to whole blood greatly improved stability of esmolol with a resultant extrapolated in vitro half-life exceeding 6 h. Placing specimens of fresh whole blood containing NaF on ice further improved the stability of esmolol, such that negligible decay occurred (Fig. 5). The esmolol esterase activity of fresh WB specimens appeared to exceed the activity observed with stored WB specimens, suggesting that esterase activity is diminished during whole blood storage.

Table 2
Deviation from nominal concentrations in three calibration curves

Nominal (ng/ml)	Plasma 1		Plasma 2		Plasma 3	
	Calculated (ng/ml)	Deviation (%)	Calculated (ng/ml)	Deviation (%)	Calculated (ng/ml)	Deviation (%)
2	1.95	−2.7	1.91	−4.8	2.27	13.7
15	15.0	0.2	15.2	1.5	16.1	7.2
25	25.9	3.5	24.9	−0.2	26.9	7.7
125	122	−2.6	135	7.9	130	4.3
250	274	9.6	250	−0.2	260	4.2
500	534	6.9	439	−12.2	485	−2.9
750	743	−1.0	805	7.4	731	−2.6
1000	951	−4.9	996	−0.4	1037	3.7

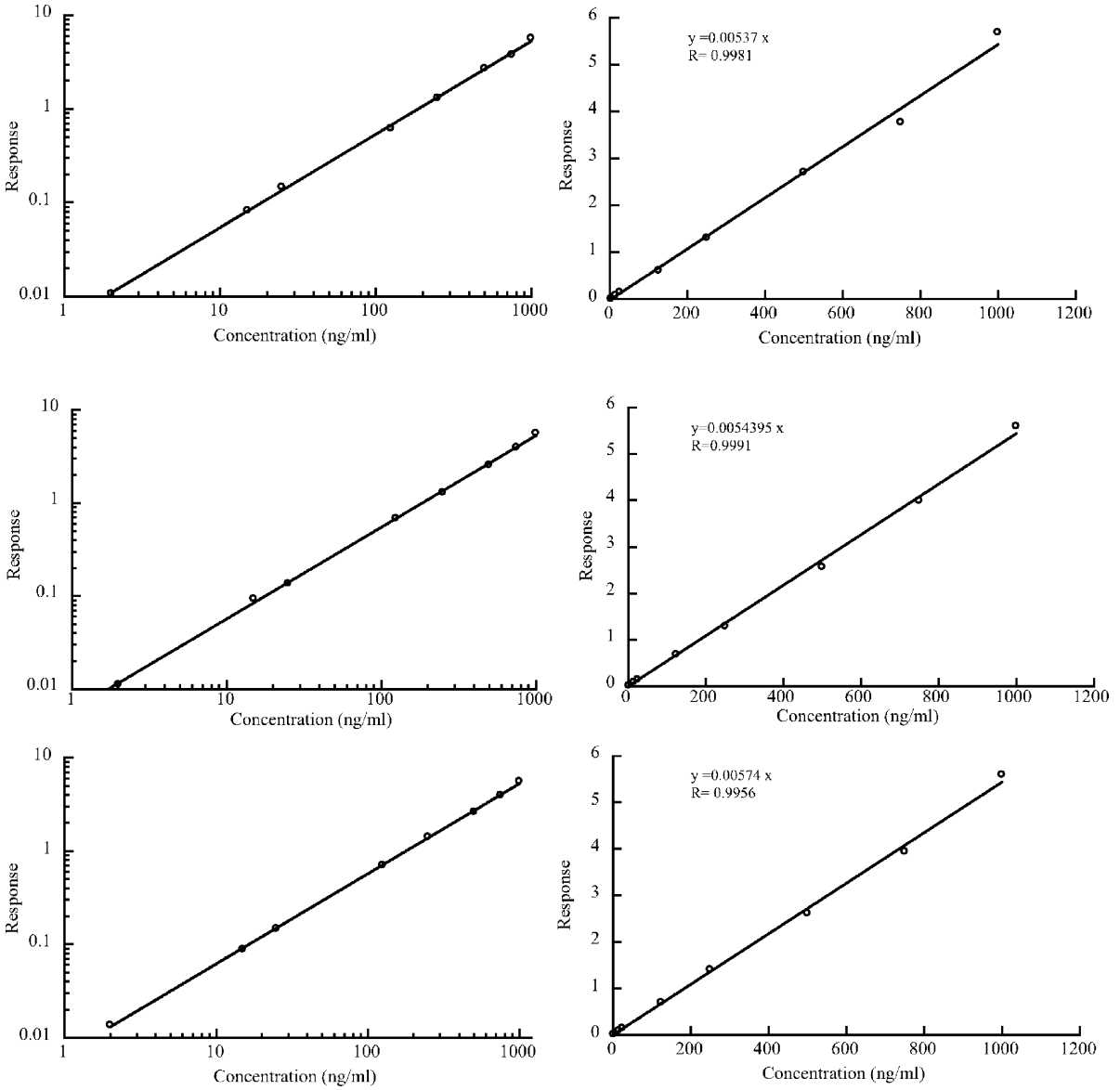

Fig. 4. Standard curves generated from three separate batches of plasma. The left column is a log–log plot to better visualize linearity at low concentrations. The right hand plot is a linear plot of response ratio vs. concentration. Table 2 reports the deviation from nominal concentrations of these standard curves.

4. Discussion

The LC–MS assay reported here accurately and precisely quantitates esmolol concentration in 200 μl plasma specimens with a limit of quantitation of 2 ng/ml. The intra-day and inter-day coefficients of variation were less than 8 and 10% respectively at all concentrations tested. With linearity up to 1000 ng/ml, the assay is well suited for use in pediatric pharmacokinetics study.

Table 3
Precision determined at four esmolol concentrations including the LOQ, low (125 ng/ml) medium (500 ng/ml) and high (750 ng/ml)

Concentration (ng/ml)	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Mean ± S.D. (ng/ml)	CV (%)
Plasma 1							
2	2.27	2.30	2.23	2.28	2.23	2.26 ± 0.03	1.3
125	133	123	122	143	138	132 ± 9	7.1
500	517	518	472	472	469	490 ± 25	5.2
750	704	809	694	708	743	731 ± 47	6.4
Plasma 2							
2	2.00	1.96	2.01	2.21	2.17	2.07 ± 0.11	5.4
125	147	133	134	139	152	141 ± 8	5.8
500	524	589	557	569	508	549 ± 33	6.0
750	717	738	739	769	733	739 ± 19	2.6
Plasma 3							
2	2.02	2.15	2.03	2.13	2.41	2.15 ± 0.16	7.5
125	115	112	114	121	130	119 ± 7	6.2
500	504	544	534	591	475	529 ± 44	8.3
750	744	702	840	751	739	755 ± 51	6.8

Table 4
The inter-day coefficient of variation was determined on three separate days using 5 determinations on each day

Concentration (ng/ml)	Mean ± S.D.	Inter-day CV (%)
2	2.16 ± 0.13	6
125	131 ± 12	9
500	519 ± 41	8
750	742 ± 40	5

As anticipated, the LOQ with this LC–MS was lower than assays that utilized UV detection methods [3,4,6,7], or gas chromatography methods [8]. Most importantly, the sample volume required for this assay was significantly smaller than all previously reported methods, and will allow for multiple sampling in infants and children.

Table 5
Accuracy was determined by replicate analysis of samples containing known amounts of esmolol

Nominal concentration (ng/ml)	Sample #1	Sample #2	Sample #3	Sample #4	Sample #5	Mean ± S.D. (ng/ml)	Deviation (%)
Plasma 1							
2	2.21	2.29	2.43	2.38	2.43	2.35 ± 0.10	17
6	7.0	6.4	6.6	6.9	7.8	6.9 ± 0.54	16
100	101	105	116	107	120	109 ± 8	10
400	386	420	390	430	401	405 ± 19	1
800	844	784	798	940	781	829 ± 67	4
Plasma 2							
2	2.08	2.04	2.19	2.56	2.09	2.19 ± 0.21	10
6	6.0	6.2	6.2	6.2	5.9	6.1 ± 0.14	2
100	107	108	102	106	135	111 ± 13	11
400	381	382	393	442	371	393 ± 28	−2
800	713	754	849	742	772	766 ± 51	−4
Plasma 3							
2	2.08	2.06	2.03	2.14	2.05	2.07 ± 0.04	4
6	5.9	5.9	5.8	6.0	6.1	5.9 ± 0.11	−1
100	100	107	100	97	102	101 ± 3	1
400	389	359	350	345	358	360 ± 17	−10
800	864	846	872	790	791	832 ± 39	4

The average value was be within 15% of the actual value except at LOQ, where it was within 20%.

Table 6
The recovery was determined by comparing the area of response of a plasma extracted specimen (100%) to an aqueous unextracted specimen

Concentration (ng/ml)	Plasma (area of response)		Aqueous (area of response)		Recovery (%)	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
25	2.77×10^5	2.79×10^5	3.27×10^5	3.19×10^5	85	87
250	2.56×10^6	2.70×10^6	2.71×10^6	2.99×10^6	94	91

Esmolol is intrinsically unstable in human blood. Due to the presence of red cell esterases esmolol undergoes rapid hydrolysis to its acid metabolite. Esmolol has one chiral center and is marketed as a racemate consisting of two enantiomers. The cardiac β-blocking activity resides in the *S*(−)-enantiomer [13]. The red cell esterase found in human blood, however, does not demonstrate stereoselectivity for either enantiomer [14]. Therefore, both enantiomers undergo ester hydrolysis, and the total plasma drug concentrations reported here are directly proportional to the concentrations of the *S*(−)-enantiomer.

The effectiveness of NaF as an inhibitor of red cell esterases was a critical issue, as ex vivo degradation of esmolol will occur if specimens are not adequately inactivated. Quon and Stamfli initially reported that NaF was an effective inhibitor of esmolol esterase [2]. Numerous other investigators then used NaF as an inhibitor when studying the pharmacokinetics of esmolol [3,4,9–11]. In 1991 Fan and Zhao reported that NaF concentrations as high as 30 mg/ml were unable to inhibit ex vivo metabolism, but detailed data were not presented [6]. Jahn et al. presented data that found that NaF (0.05 μM) increased the in vitro half-life of esmolol twofold at room temperature [12]. One finding presented here that may in part explain the variability reported by investigators on the ability of NaF to inactivate red cell esterases is that the source of whole blood used in experiments impacts the activity of the esmolol esterase. Whole blood obtained from the blood bank appeared to have diminished activity compared with fresh blood. In addition, we found that when using the previously described HPLC-UV method [3], specimens from a subset of volunteers contained a UV chromatographic peak, distinguishable with diode array detection, that co-eluted with the carboxylic acid metabolite of esmolol (data not shown). Thus, it is possible that presence of this

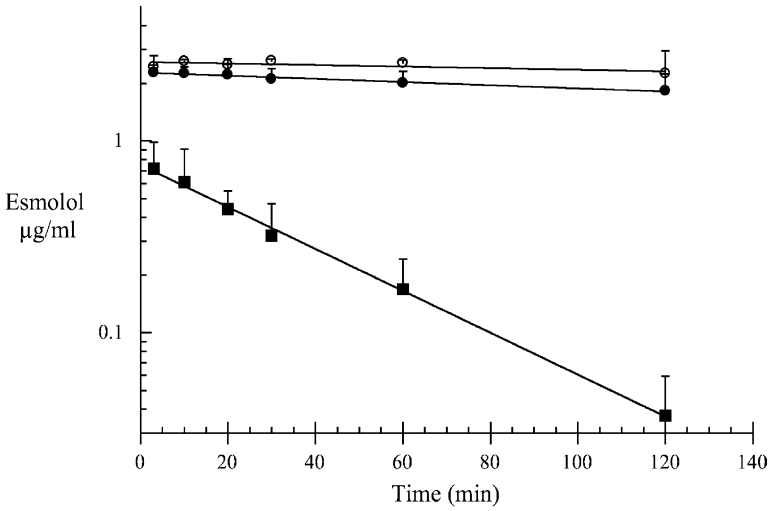

Fig. 5. Plasma concentrations of esmolol obtained from whole blood at room temperature (closed squares), whole blood + NaF at room temperature (closed circles), and whole blood + NaF on ice (open circles). Values are mean ± S.D.

peak may have been interpreted as evidence of significant esmolol instability by investigators. Although NaF does not completely inhibit the esterase activity present in fresh whole blood at room temperature, the rate of ex vivo degradation is low. The combination of NaF addition and rapidly placing specimens on ice effectively eliminates ex vivo decay. Specimens handled in this manner are stable for at least 2 h.

In vitro radioligand binding demonstrated that esmolol has a 34-fold higher affinity for β_1 receptors than for β_2 receptors. Its metabolite has a very low and nonspecific affinity for both receptor subtypes. The metabolite is 400-fold less potent than esmolol at the β_1 receptor [12]. As such, the pharmacodynamic activity of esmolol is short lived, and clinicians readily extinguish β-blockade by discontinuation of the drug infusion.

Since the original report that utilized methylene chloride for esmolol extraction that formed the basis for the extraction used here, the use of this organic solvent in research laboratories has continued to decrease. For this reason, we also explored use of ethyl acetate as an alternative to methylene chloride for extraction. Standard curve slope, linearity, and quality control specimen analyses were acceptable and virtually identical to the results obtained with methylene chloride (data not shown). It thus appears reasonable to substitute ethyl acetate for methylene chloride when performing this assay.

Acknowledgements

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