Journal of Chromatography, 441 (1988) 197–205
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1315

DETERMINATION OF CLENBUTEROL AND MABUTEROL IN EQUINE PLASMA BY ION-PAIR LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

CHROMATOGRAPHIC AND ELECTROCHEMICAL CHARACTERISTICS

G. ALI QURESHI*,*
Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Swedish Agricultural University, BMC, Box 573, S-751 23 Uppsala (Sweden)
and
ALF ERIKSSON
Department of Analytical Chemistry, Institute of Chemistry, University of Uppsala, Box 531, S-751 21 Uppsala (Sweden)

SUMMARY

A method for the routine determination of the β-adrenergic drugs clenbuterol and mabuterol in equine plasma has been developed. The drugs were isolated from alkalinized plasma by liquid–liquid extraction. The organic phase was evaporated to dryness and the residue was dissolved in the mobile phase prior to injection. The recoveries were 98% and 95% for clenbuterol and mabuterol, respectively.

The drugs were separated by reversed-phase high-performance liquid chromatography and quantitated by a use of a coulometric detector set at +0.75 V *vs.* the internal reference electrode. The influence of pH and amounts of organic modifier and ion-pairing agent on the retention times was investigated. The relationship between peak current and concentration was linear up to 1 μg/ml for both compounds. The limits of detection were 0.5 ng/ml for clenbuterol and 2 ng/ml for mabuterol with a signal-to-noise ratio of 3. A brief discussion of the electrochemistry of the compounds is given.

INTRODUCTION

Basic drugs are often used in the treatment of ill or injured race horses in training, including stimulants, depressants, anaesthetics and tranquillizers. As the drugs not only have therapeutic effects but also effects on the racing performance of

* Present address: Department of Renal Medicine, Karolinska Institute Huddinge University Hospital, S-141 86 Huddinge, Sweden.

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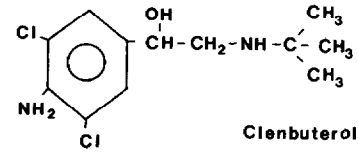
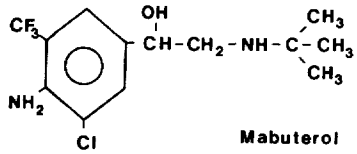
the horses, they are usually listed as dopants by different official horse-racing organizations[1]. Suitable methods for the routine determination of these listed compounds are therefore necessary, both for the clearance of horses after legal treatment and for the detection of illegal use.

Two drugs of this type are clenbuterol (I) and mabuterol (II) (Fig. 1). Clenbuterol was listed by the Association of Official Racing Chemists in the USA as a dopant in 1985[1]. These drugs are β-adrenergic agents[2–5] and are used as bronchial dilators for the treatment of chronic, obstructive pulmonary disease and bronchospasm associated with allergies, infection and exercise[4–9]. Clenbuterol has been shown to possess prolonged duration of action, good oral bioavailability and a pronounced effect on the secretion and transport of mucous by stimulating bronchial secretory and ciliated cells[10,11].

Pharmacokinetic studies of the compounds have been made with radiological methods[4,5]. However, for routine analysis this approach is not suitable, as it requires radioactively labelled compounds. Alternative methods are gas chromatography (GC)[12], thin-layer chromatography (TLC) with UV or mass spectrometric (MS) detection[13] and high-performance liquid chromatography (HPLC) with UV[14,15] or electrochemical detection[16]. However, most of the methods mentioned above suffer from different drawbacks. GC requires derivatization of the analytes owing to their polar nature. TLC–UV and HPLC–UV methods have detection limits that are too high for quantitation of the drugs in plasma after normal drug administration and TLC–MS is too expensive for most laboratories. Therefore, the most promising approach seems to be the use of HPLC with electrochemical detection (ED), as the compounds contain an electroactive aromatic amino group. Aromatic amines, such as substituted anilines, are easily oxidized at a carbon electrode[17,18]. ED has already been successfully utilized by Diquet *et al.*[16] for the determination of I in mouse



Clenbuterol (I)



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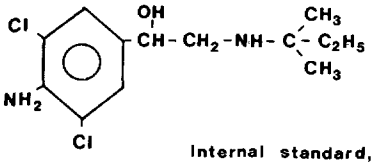

Internal standard, NAB 760 (III)

Fig. 1. Structures of the compounds tested.

plasma. Unfortunately, their method suffers from a low recovery and is incapable of the simultaneous measurement of different β-2-antagonists.

In this paper we describe the chromatographic and electrochemical properties of I and II. We have studied the influence of pH and the concentration of the organic modifier and ion-pairing agent on the retention of the compounds. Optimization of the extraction step has also been performed. Cyclic voltammetry was utilized to determine the oxidation potentials of the compounds and to study the adsorption on carbon electrode surfaces. The results from these studies were used to develop a method for the routine analysis of horse plasma with drug concentrations in the ng/ml range.

EXPERIMENTAL

Apparatus

The HPLC system consisted of an LDC/Milton Roy Constametric pump (Model G III) equipped with a pulse damper, a U6K injector with a 50μl sample loop from Rheodyne (Catati, CA, U.S.A.), a pre-column (4 × 4 mm I.D.) and an analytical column (125 × 4 mm I.D.), both packed with 7-μm LiChrosorb (Select B) C_{18} (E. Merck, Darmstadt, F.R.G.). An ESA coulometric detector (Model 5011) was used connected to a ESA Coulochem (Model 5100A) potentiostat (Environmental Science Assoc., Bedford, MA, U.S.A.). The working electrode was monitored at +0.75 V *versus* the internal reference electrode. The chromatograms were recorded on a strip-chart recorder.

The voltammetric experiments were performed with equipment based on an ABC-80 personal computer (Luxor, Motala, Sweden), which generates the potential programme applied to the potentiostat[19–21]. A conventional three-electrode system with a glassy carbon or carbon paste as an indicator electrode was used. The counter electrode was a platinum wire, and a saturated calomel electrode (SCE) was used as the reference electrode. The measurements were made in a 5-ml Metrohm electrochemical measuring cell and all voltammograms were recorded on an *x–y* recorder (Model 4077A, Hewlett-Packard, Mountain View, CA, U.S.A). The glassy carbon electrode (GCE) was polished daily with a slurry of 0.075-μm alumina and rinsed four times with water in an ultrasonic bath. Removal of adsorbed compounds was achieved by rubbing the electrode against tissue paper, soaked with dichloromethane. The electrode was pretreated at +1.55 V *vs.* Ag–AgCl in 50 m*M* sulphuric acid until cyclic voltammetry yielded a peak separation of 100 mV for hydroquinone (1 m*M* in 50 m*M* sulphuric acid) at a scan rate of 0.10 V/s.

New surfaces on the carbon paste electrode (CPE) were obtained according to ref. 22.

Chemicals and Standards

Clenbuterol (I), mabuterol (II) and the internal standard NAB 760 (III) (Fig. 1) were kindly provided by Boehringer (Ingelheim, Switzerland). Sodium 1-heptanesulphonate was obtained from Fison Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals were of analytical-reagent grade and obtained from E. Merck. Standard solutions of I, II and III (10 μg/ml) were prepared in doubly distilled water. The addition of a few drops of 0.1 *M* formic acid increased the stability of the standard

solutions. All solutions were stored at +4°C and remained stable for at least 1 month. Calibration standards were prepared by further dilution of the standards to concentrations 5, 10, 20, 30 and 50 ng/ml.

The optimized mobile phase was phosphate buffer (pH 4.0–4.1, ionic strength 0.1)–acetonitrile (77:23, v/v). Sodium 1-heptanesulphonate was added to a concentration of 0.2 m*M* and the final solution was filtered through a 0.2-μm membrane and degassed in an ultrasonic bath for 1 h before use. The mobile phase was prepared fresh daily.

Voltammetric experiments were performed using both acetate and phosphate buffers of pH 4.0. The concentration of the compounds in pure buffer solutions was 0.15 mg/ml, corresponding to 0.43, 0.48 and 0.46 m*M* for I, II and III, respectively. The experiments were repeated after successive addition of acetonitrile to the solutions. The amounts of acetonitrile in phosphate buffer were 10, 20, 25, 30 and 40% (v/v) and in acetate buffer 25 and 40% (v/v).

Treatment of blood samples

Blood samples from healthy horses were collected in heparin-containing test-tubes. Plasma was obtained by centrifuging the blood samples at 1500 *g* for 10 min. The plasma samples were stored at −20°C until the time of analysis. No drugs were administered to the animals for 2 weeks prior to sample collection.

Analytical procedure

A 1-ml volume of 0.25 *M* sodium hydroxide solution, 100 μl of III (50 ng/ml) and 1 ml of plasma or standard were transferred into a 10-ml test-tube and mixed for 2–3 min. The compounds were extracted into 5 ml of diethyl ether–2-butanol (9:1) by shaking the tube for 10 min. After centrifugation at 1500 *g* for 10 min, 4 ml of the organic phase were transfered into a test-tube and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of mobile phase by agitating the tube for 2 min on a vortex mixer. The final sample solution was stored at +4°C overnight. The samples were allowed to attain room temperature before injection of a 50-μl aliquot into the chromatograph. Storing the samples at 4°C overnight reduced baseline disturbances and the number of interfering peaks from the plasma. Standard, samples and blank plasma, spiked with different amounts of the drugs, were all treated according to this procedure. The concentration of each drug was calculated from the peak-height ratio between I or II and III.

RESULTS AND DISCUSSION

Extraction of compounds

In order to optimize the extraction procedure, various solvents were investigated as the organic modifier. Previous studies have shown that the extraction of salbutamol, which is a structural analogue of I, is difficult owing to its hydrophilic nature in acidic media[23,24]. To overcome this difficulty, we used diethyl ether–2-butanol (9:1) to extract the drugs from alkalinized plasma, as described under Experimental. The procedure affords recoveries of 98% and 95% of I and II, respectively, with a standard deviation of 2% for both compounds. This extraction procedure also accomplished a complete separation of the compounds from plasma proteins. Hence, the use of classical deproteination methods was not necessary.

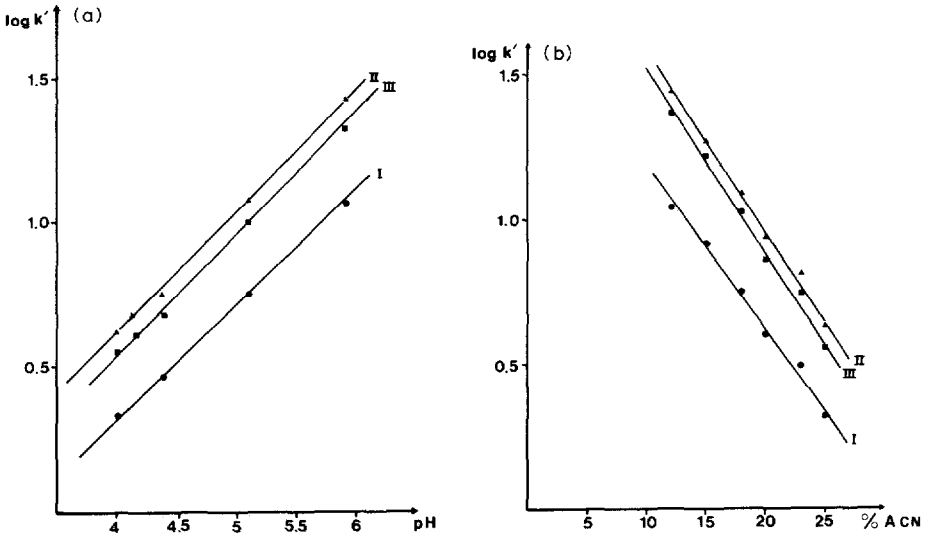

Fig. 2- (a) Effect of eluent pH on log k' for clenbuterol (I), mabuterol (II) and internal standard (III). For other experimental conditions, see text. (b) Effect of acetonitrile (ACN) content in the mobile phase on log k' for the compounds tested under otherwise identical experimental conditions.

Chromatographic experiments

The capacity factor is related to the degree of protonation of the amino groups in the molecule. Therefore, the retention of the compounds depends on the pH of the mobile phase, as shown in Fig. 2a. The content of acetonitrile is another parameter that influences the retention. A higher content of acetonitrile increases the eluting power of the mobile phase (decreasing log k') and thus decreases the retention times (Fig. 2b).

Often an unknown peak (x) was present in the plasma sample and this peak interfered with the peak of I. In order to separate the unknown substance from I, a hydrophobic ion-pairing compound was added to the mobile phase. As the compounds studied are positively charged at pH < 5, a negatively charged agent, sodium 1-heptanesulphonate, was used. The retention of positively charged substances is therefore increased without affecting the retention of negatively or uncharged compounds. The effect of different concentrations of the ion-pairing agent on the capacity factor is shown in Fig. 3. A concentration of 0.2 mM was found to be sufficient to separate peak x from peak I. In Fig. 4, chromatograms are presented for (a) a standard mixture, (b) blank plasma spiked with III and (c) plasma containing I, II and III.

The calibration graphs were plotted as peak-height (h) ratios between I or II and III against concentration (c) according to the following equations:

$$h(\mathrm{I})\ /h(\mathrm{III}) = 0.21c(\mathrm{I}) + 0.08 \quad (1)$$

$$h(\mathrm{II})/h(\mathrm{III}) = 0.05c(\mathrm{II}) + 0.03 \quad (2)$$

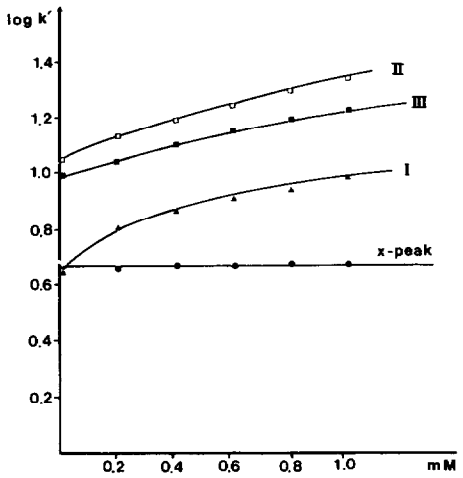

Fig. 3. Effect on log k' of different concentrations of sodium 1-heptanesulphonate in the mobile phase. I = Clenbuterol; II = mabuterol; III = NAB 760; x = interfering peak.

For eqn. 1, $r^2 = 0.9995$, $n = 8$, concentration range = 0.5–10 ng/ml, and for eqn. 2, $r^2 = 0.994$, $n = 5$, concentration range = 2–10 ng/ml. The concentration of III was 5 ng/ml. The calibration graphs are linear up to 1 μg/ml of I and II for both

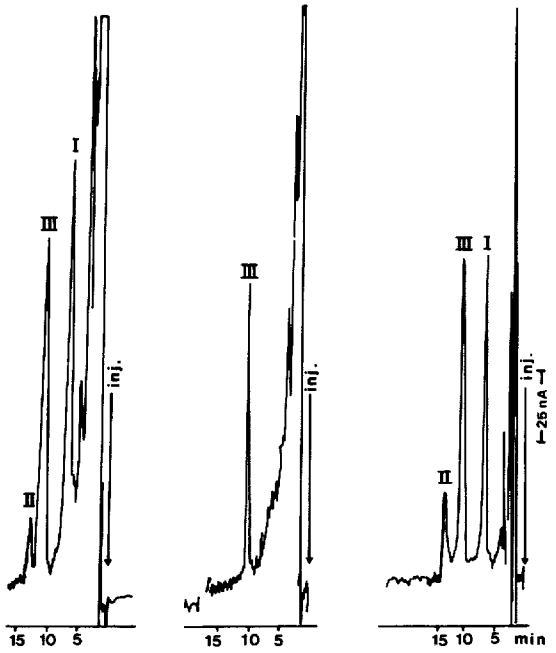

Fig. 4. Typical chromatograms of (right) standard mixture (10 ng/ml) of I, II and III, (middle) blank plasma spiked with NAB 760 (10 ng/ml) and (left) plasma sample containing 10 ng/ml of I, II and III.

TABLE I

RESULTS OF CYCLIC VOLTAMMETRY

Carbon paste electrode (CPE); area, 28 mm²; scan rate, 100 mV/s. Standard deviations are given in parentheses.

Compound	*Buffer**	*pH*	E_p *(mV)*	$E_p/2$ *(mV)*	I_p *(μA)*
I	Phosphate	3.97	1062 (3)	988 (5)	89 (4)
I	Phosphate	4.0	1095 (2)	1037 (2)	53 (1)
III	Phosphate	4.06	1095 (2)**	983 (2)	70 (4)
I	Acetate	3.98	1056 (3)	971 (2)	96 (2)
II	Acetate	4.02	1093 (6)	1032 (4)	51 (1)
III	Acetate	4.03	1095 (7)**	974 (4)	72 (1)

* 0.05 *M*.

** No well defined peak in voltammogram.

standards and spiked blank plasma. The limits of detection (signal-to-noise ratio = 3) were 0.5 and 2 ng/ml for I and II, respectively.

Voltammetry

The voltammetric experiments were performed in order to investigate the electrochemical properties of these compounds. At both the CPE and GCE it was found that these compounds were oxidized irreversibly, although large positive potentials were required. Both phosphate and acetate buffers yielded approximately the same results. The results at the CPE are summarized in Table I. Compounds I and III exhibited nearly the same $E_p/2$ values, which is to be expected as they have very similar structures (Fig. 1). The large positive potentials required are probably due to the electrophilic substituents on the aromatic ring, as was shown by Bacon and Adams[17] for aniline and chlorinated anilines. The higher $E_p/2$ value for II is probably a consequence of the more electrophilic CF_3 substituent in this compound.

The irreversible oxidation of the compounds, *i.e.*, the absence of reduction waves on the reverse scan, is a consequence of a chemical follow-up reaction of the oxidation product (EC or ECE process). This is also supported by the appearance of small reduction and oxidation waves in subsequent scans at considerably lower positive or negative potentials on both carbon electrodes (results not shown). The oxidation of different anilines is known to show the similar behaviour[17,18].

Para-substituted anilines often undergo "head-to-tail" radical coupling, which results in the loss of the *para* substituent on one molecule and the formation of different aminodiphenylamines. These are often oxidizable at less positive potentials than the original aniline[17,18], and the additional waves for the compounds studied may be explained by a similar process.

The compounds show several signs of adsorption on the carbon electrodes. Both pre- and post-waves are observed, especially when scan rates higher than 100 mV/s are employed. These waves often interfere with the determination of various parameters from the voltammograms such as E_p, $E_p/2$ and I_p, thus increasing the uncertainty of these parameters. Further, adsorption of reaction products blocks the active site of the electrodes. This is evidenced by lower peak currents, changes in

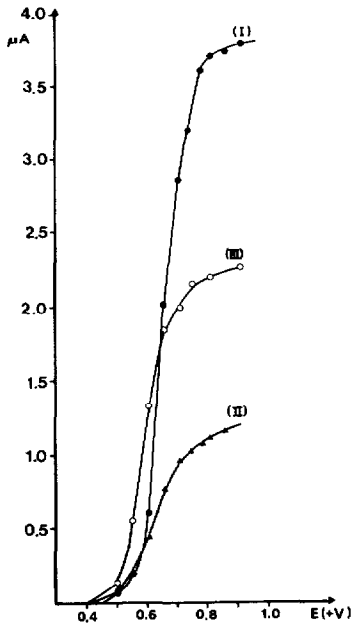

Fig. 5. Hydrodynamic voltammogram of 0.1 μg/ml of clenbuterol (I), mabuterol (II) and NAB 760 (III), obtained with the HPLC system, with phosphate buffer (pH 4.0)–acetonitrile (77:23, v/v) as mobile phase at a flow-rate of 0.7 ml/min. For other experimental conditions, see text.

wave shapes and increased E_p and $E_p/2$ in the subsequent scans. The effects are relatively pronounced on the GCE, on which the adsorption of the reaction products in some instances causes complete inhibition of the current response. However, the adsorption can be reduced by addition of acetonitrile, and when the acetonitrile content is about 30–40%, the adsorption has almost disappeared at the GCE.

Detection system

The relatively large electrode surface of the coulometric detector reduces the influence of adsorption on the detector response. The addition of more than 20% of acetonitrile to the mobile phase also contributes to minimizing the adsorption on the electrode, as discussed above. Further, the time required to poison the electrode surface completely is also lengthened by the much lower concentrations in the flow system compared with those in the voltammetric experiments. However, a slow decrease in the response could still be observed in the system described. The most rapid method for restoring the detector response is to change the potential of the detector to −1.5 V for 5–6 min while the mobile phase continues to flow. This procedure was applied when the detector response had dropped to about 75% of that of a fresh electrode.

Fig. 5 shows the hydrodynamic voltammograms of the analytes in the chromatographic system with phosphate buffer (pH 4.0)–acetonitrile (77:23, v/v) as mobile phase. A working potential of +0.75 V was chosen for the quantitation of the compounds. This choice eliminates baseline distrubances and offers sufficient sensitivity.

CONCLUSIONS

The proposed method, based on HPLC with coulometric detection, offers a simple and sensitive method for determining I and II in the ng/ml range in equine plasma in only 1 ml of sample. The voltammetric experiments show that the compounds are oxidizable at about +1.1 V *vs.* SCE at carbon electrodes. However, the electrode response is complicated by both chemical reactions and adsorption of the oxidation products. Addition of acetonitrile to the electrolyte reduces the adsorption of the compounds investigated on the GCE.

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