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Comparison of two extraction methods for determination of propranolol and furosemide in human plasma by mixed-mode chromatography

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Abstract

An isocratic high performance liquid chromatographic method is described for the determination of the β-adrenergic blocking drug, propranolol, and the diuretic, furosemide, in human plasma. The two compounds and the internal standard were extracted from plasma using a two-step extraction technique. Propranolol and pindolol (internal standard) were first extracted from alkaline plasma into diethyl ether; this was followed by extraction of furosemide into acidified ether: hexane (65:35). The two extracts were then combined and evaporated under nitrogen, and the reconstituted residues were analysed on a C18/SCX reversed-phase/cation exchange column with a mobile phase of acetonitrile: 0.1 M sodium acetate pH 4 (33:67). The drugs and the internal standard were detected by UV absorption at 230 nm. The drugs were also extracted from plasma by a column-switching technique utilizing a ten-port valve. The drug compounds were retained on a C18 pre-column. A comparison of RSD for within-batch (intra-assay) and between-batch (inter-assay) runs for both methods was carried out, the liquid/liquid extraction method giving better recovery values. The calibration graphs were linear from 25–300 ng ml^{-1} for furosemide and 50–400 ng ml^{-1} for propranolol. Recovery values were $> 90.0\%$ by liquid/liquid extraction and $> 76.0\%$ by column switching.

Keywords: Mixed-mode chromatography; Column switching; Propranolol; Furosemide; Plasma

1. Introduction

Propranolol (Fig. 1) is a β-adrenergic blocking drug that has found wide application for the treatment of cardiac arrhythmia, sinus tachycardia [1], angina pectoris and hypertension. It has also been suggested for use for a number of other conditions including dysfunctional labour [2] and anxiety [3]. It has been found necessary on occasion to administer the diuretic furosemide to overcome some of the side-effects of propranolol. Furosemide (Fig. 1) is considered to be a short-acting loop diuretic. It exerts its major effect by inhibiting sodium reabsorption in the proximal convoluted tubule and the loop of Henle. Its

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major uses are in acute or chronic renal failure, congestive heart failure and liver cirrhosis [4,5].

To date there have been a number of techniques which have been used for the determination of propranolol, including GC–MS [6], MS [7] and HPLC [8–10]. The principal methods for determination of furosemide have been HPLC [11,12] and GC–MS [13]. Cline-Love et al. [14], developed a micellar liquid chromatographic technique which allowed determination of propranolol and furosemide in urine by micellar chromatography using a Hypersil C18 column and Brij 35 as the surfactant. The micellar mobile phase was optimized by varying the pH and concentration of Brij 35; depending on these conditions either propranolol or furosemide could be determined. To date no other method has been reported that allows the simultaneous determination of furosemide and propranolol.

The aim of this work was to investigate the use of a mixed-mode stationary phase for the simultaneous determination of propranolol and furosemide in biological fluids. Development of the chromatography and extraction procedures from first principles are also described. Two sample clean-up procedures were examined; liquid/liquid extraction and column-switching. Each of these methods was validated and a comparison of the two methods was carried out.

2. Experimental

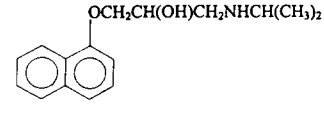
2.1. Reagents and solvents

Propranolol, pindolol and furosemide were obtained from Sigma Chemical Co. (Poole, UK). HPLC grade acetonitrile, diethyl ether, hexane and water were obtained from Labscan Analytical Sciences (Dublin, Ireland). Analar grade sodium acetate, acetic acid and sodium hydroxide were supplied by Merck (Darmstadt, Germany). Deionised water was obtained using an Elgastat spectrum water purification unit. A small pooled human plasma sample was obtained from a volunteer by drawing blood into evacuated tubes containing heparin as anti-coagulant. These were then centrifuged at 3000 rev min^{-1} for 5 min and the upper plasma layer was grently removed and stored at $-18°C$ until required for assay puposes.

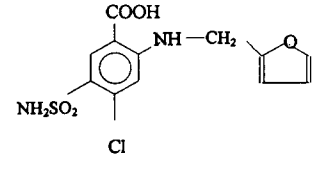
2.2. Standards

Stock solutions were prepared by dissolving the appropriate amount of analyte in methanol to yield a solution of concentration 1 mg ml^{-1}. A set of calibration standards were prepared by dilution of the stock solution with deionised water. The furosemide standards covered the range 25–200 ng ml^{-1} and the propranolol standards covered the range 50–400 ng ml^{-1}. A stock solution of pindolol (the internal standard) was made to a concentration of 100 μg ml^{-1} in methanol. A working solution of 20 μg ml^{-1} was prepared by dilution of the stock solution with deionised water.

Propranolol



Furosemide



Pindolol

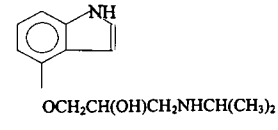


Fig. 1. Chemical structures of propranolol, furosemide and pindolol (the internal standard).

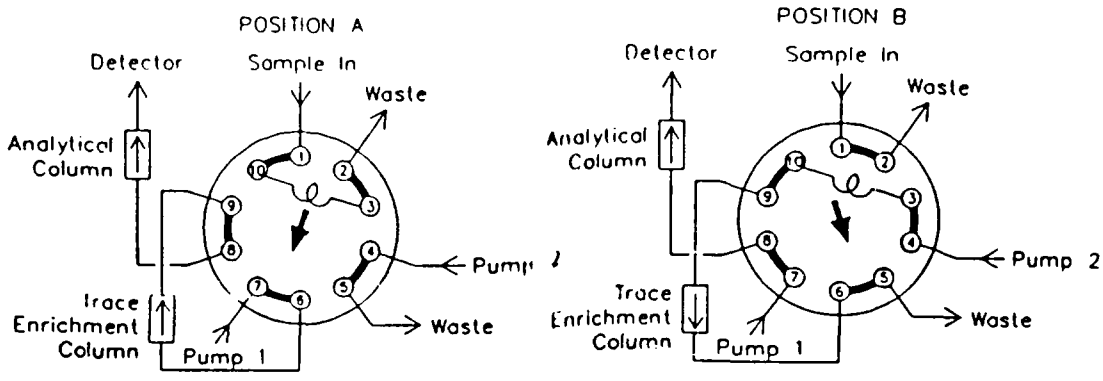


Fig. 2. Diagram of 10-port injection valve.

2.3. Plasma standards

Aliquots of blank plasma were spiked with stock solutions to produce the required concentrations of the two drug components and the internal standard.

2.4. Instrumentation and operating conditions

Furosemide, propranolol and pindolol were separated on a C18/SCX (5 μm) reversed-phase/cation exchange column (250 mm × 4.6 mm i.d.) supplied by Shandon Scientific Ltd. (Chesire, UK). The mobile phase was 100 mM sodium acetate (pH 4.0)–acetonitrile (67:33) delivered at a flow rate of 1.0 ml min^{-1} by a Waters 510 HPLC pump (Waters Associates, Milford, MA). Injection was by a Rheodyne (Cotati, CA) injection valve fitted with a 20 μl loop. The analytes were detected by ultraviolet absorption at 230 nm using a Waters Model 486 spectrophotometric UV detector (Waters). The resulting chromatograms were recorded on an integrator (Waters 746 Data Module). For the purpose of column-switching a Waters 501 pump and an extraction column were connected to the analytical assembly via a ten-port switching-valve (Fig. 2). The extraction column (10 mm × 1.5 mm i.d.) was packed with Hypersil (Shandon) C18 (30 μg) material. Under the described chromatographic conditions the mean retention times for the elution of furosemide, pindolol and propranolol were 5.70, 7.30 and 12.50 min respectively.

3. Procedures

3.1. Extraction method 1 (liquid/liquid extraction)

As furosemide is acidic and propranolol is basic, a single extraction step will not permit the simultaneous recovery of the two compounds. Therefore it is neccessary to carry out a double extraction; this involves first extracting the furosemide from acidified plasma and then extracting the propranolol and pindolol from alkaline plasma. A series of plasma standards were

Table 1
Intra-assay for liquid/liquid extraction

Amount added (ng ml^{-1})	Mean amount found ± SD	%RSD
Propranolol		
50	54 ± 4	7
100	100 ± 11	11
150	135 ± 6	4
300	318 ± 11	4
500	493 ± 8	2
Mean RSD = 5%		
$y = 0.00015 + 0.99929x$, $r = 0.995$		
Furosemide		
50	53 ± 8	14
100	90 ± 19	21
150	161 ± 19	12
250	246 ± 4	4
300	303 ± 8	8
Mean RSD = 12%		
$y = -0.17593 + 1.00433x$, $r = 0.995$		

Table 2
Inter-assay for liquid/liquid extraction

Amount added (ng ml^{-1})	Mean amount found ± SD	%RSD
Propranolol		
50	56 ± 6	11
100	99 ± 5	5
150	136 ± 9	7
300	312 ± 16	5
500	494 ± 6	1
Mean RSD = 6%		
$y = 0.63692 + 0.99425x$, $r = 0.997$		
Furosemide		
50	58 + 8	14
100	91 + 5	5
150	147 + 28	19
250	239 + 17	7
300	294 + 19	7
Mean RSD = 10%		
$y = 3.39640 + 0.95480x$, $r = 0.996$		

prepared containing furosemide, pindolol and propranolol and these were submitted for extraction. The extraction procedure was based on a method by Lindström et al. [15]. Furosemide was extracted by adding 20 μl of 2.0 M acetic acid to 125 μl of spiked plasma, and vortex-mixing each tube for 30 s. After the addition of 1 ml diethylether:hexane (65:35), the drug was extracted by vortex mixing for 150 s. Following centrifugation at 1000*g* for 15 min at 4°C, the upper organic layer (800 μl) was transferred into a clean polypropylene Eppendorf tube. The plasma was subjected to further extraction to remove basic components following a procedure based on a method by Kerremans et al. [16]. In this procedure, the basic components were extracted by adding 50 μl 1 M NaOH to the plasma and vortex mixing for 60 s. After addition of 1 ml of diethylether to all tubes, they were then centrifuged at 1000*g* for 15 min at 4°C. The upper organic layer (800 μl) was removed and added to the acidic layer. The combined layers were evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of mobile phase and a 20 μl aliquot was injected for chromatography.

3.2. Extraction method 2 (column-switching)

The ten-port valve used in this part of the study is shown in Fig. 2. The spiked plasma sample was introduced via the injector port and swept onto the extraction column by water delivered by pump B. The drug components were selectively retained by the packing material in the extraction column, while the endogenous plasma components were eluted to waste. Upon switching the valve the mobile phase was diverted in a backflush mode via the extraction column, where it desorbed the drugs and swept them onto the analytical column for separation.

3.3. Calibration and calculation

Evaluation of the assay was carried out by the construction of a five-point calibration graph covering the concentration ranges 25–200 ng ml^{-1} (furosemide) and 50–400 ng ml^{-1} (propranolol) in plasma for the liquid/liquid extraction method. The concentration ranges for the column-switch-

Table 3
Intra-assay for column switching

Amount added (ng ml^{-1})	Mean amount found ± SD	%RSD
Propranolol		
50	49 ± 10	21
100	100 ± 14	14
200	201 ± 23	12
300	295 ± 22	7
400	416 ± 15	4
Mean RSD = 11%		
$y = -4.74207 + 1.03271x$, $r = 0.998$		
Furosemide		
25	28 + 6	21
50	52 + 11	22
100	104 + 19	18
150	157 + 12	8
200	202 + 5	2
Mean RSD = 14%		
$y = 2.61652 + 1.00729x$, $r = 0.999$		

Table 4
Inter-assay for column-switching

Amount added (ng ml^{-1})	Mean amount found ± SD	%RSD
Propranolol		
50	45 ± 8	17
100	102 ± 19	18
200	210 ± 15	7
300	294 ± 14	5
400	401 ± 20	5
Mean RSD = 10%		
$y = 0.30470 + 0.99991x$, $r = 0.998$		
Furosemide		
25	24 ± 5	22
50	58 ± 14	24
100	95 ± 9	9
150	150 ± 16	11
200	197 ± 13	7
Mean RSD = 14%		
$y = 2.44341 + 0.97303x$, $r = 0.996$		

ing method were 25–200 ng ml^{-1} for furosemide and 50–500 ng ml^{-1} for propranolol. The slope and intercept of the calibration graphs were determined through linear regression of the drug-to-internal standard peak-height ratio vs. drug concentration plot. Individual peak-height ratios were then interpolated on the calibration graphs to determine values of concentration found as compared to concentration added.

4. Results and discussion

4.1. Chromatographic conditions

It is often necessary in drug management programmes to co-administer a number of components, in order to overcome side-effects. Since propranolol and furosemide are occasionally co-administered, the aim of this work was to develop a suitable method for the simultaneous determination of furosemide and propranolol in plasma. The initial step was to select the mobile phase conditions and selection was guided by previous work in this area [17]. The proportions of the buffer and pH were varied until maximum separation was achieved for the components and the final mobile phase contained acetonitrile: sodium acetate (pH 4) (67:33). This system was capable of adequately resolving furosemide, pindolol and propranolol from endogenous components in the plasma (Fig.3).

4.2. Recovery

Recovery of furosemide/propranolol from plasma was measured by calculating the precentage difference between the peak heights of extracted standards and those of the authentic (unextracted) standards in the relevent concentration range. Using this method the mean recovery by the liquid/liquid extraction method for furosemide from plasma was found to be 90.75% and for propranolol to be 90.08%. The mean recovery by the column switching extraction method for furosemide from plasma was found to be 76.32% and for propranolol to be 90.80%.

4.3. Selectivity

A number of drugs were investigated as potential interferants in the method and these included nitrazepam, clonazepam and quinalbarbitone. None of these were found to interfere.

4.4. Assay validation

Each of the methods developed for the simultaneous determination of propranolol and furosemide were validated over the concentration ranges shown in Tables 1–4.

4.5. Precision

The data presented in Tables 1 and 2 demonstrate the inter- and intra-assay variation in the liquid/liquid extraction method. The data presented in Tables 3 and 4 demonstrate the inter- and intra-assay variation in the column-switching method. Inter-assay variation was assessed singly in four replicate runs. Intra-assay variability was determined in quadruplicate over the same concentration range. The precision of the method (as

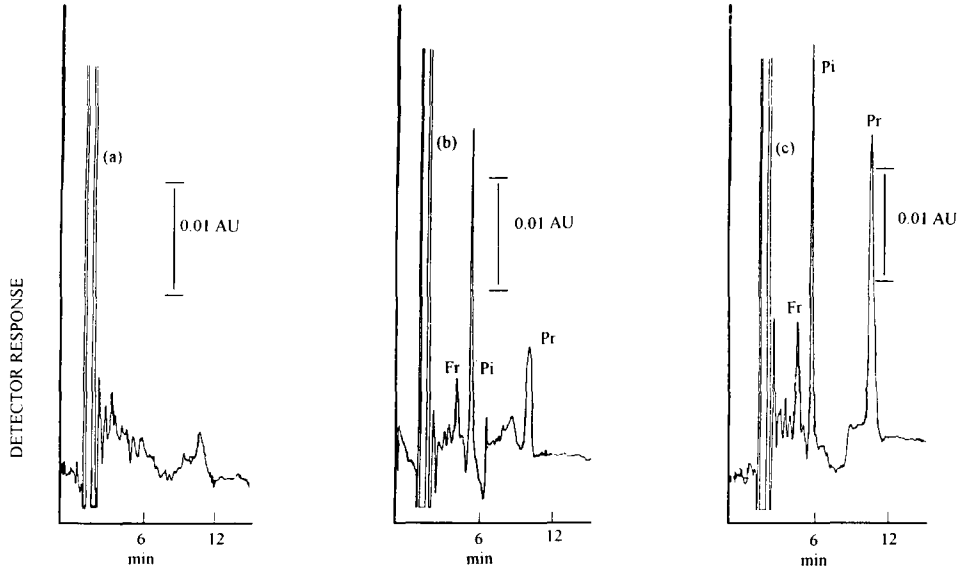


Fig. 3. Chromatograms showing (a) blank plasma, (b) the lowest concentration and (c) the highest concentration following liquid/liquid extraction. Compounds were separated on a Hypersil C18/SCX column using a mobile phase of 100 mM sodium acetate (pH 4)–acetonitrile (67:33 v/v) delivered at a flow rate of 1.0 ml min^{-1}.

expressed by mean RSD) was determined for analyte to internal standard peak-height ratios when interpolated as unknowns on the regression lines. For inter-assay variation, peak-height ratios were interpolated on the four regression lines generated from the four replicate runs. For intra-assay variation, peak-height ratios were interpolated on a single regression line generated from the quadruplicate run. The mean RSDs for each method are given in Tables 1–4.

4.6. Linearity and accuracy

Linearity is defined by the correlation coefficient of the regression line, and accuracy is defined by the percentage difference between "added" and "found" concentrations for intra-assay values presented in Tables 1–4. The correlation coefficient of the regression line for the mean intra-assay value was 0.995 or better in all cases.

5. Conclusion

This study shows that a mixed-mode C18/SCX column can find application in the simultaneous determination of strongly basic and acidic compounds. The HPLC method described is capable of simultaneously determining propranolol and furosemide extracted from human plasma. It was found that they could be adequately separated on a mixed-mode column in less than 15 min. Pindolol was the internal standard used. Liquid/liquid extraction and column-switching techniques were developed for the determination of propranolol and furosemide from plasma samples. Each of the methods was validated in terms of RSD and recovery, linearity and accuracy. The column-switching method has the advantage of being less tedious and time-consuming than the liquid/liquid extraction method, although an interfering peak was observed in the chromatograms using this method of extraction (Fig. 4). The mean recovery value for furosemide obtained by this method was much lower than the mean recovery value obtained by the liquid/liquid extraction method. The liquid/liquid extraction yields better reproducibilities and
fewer interferences and hence would find more application as a routinely used assay.

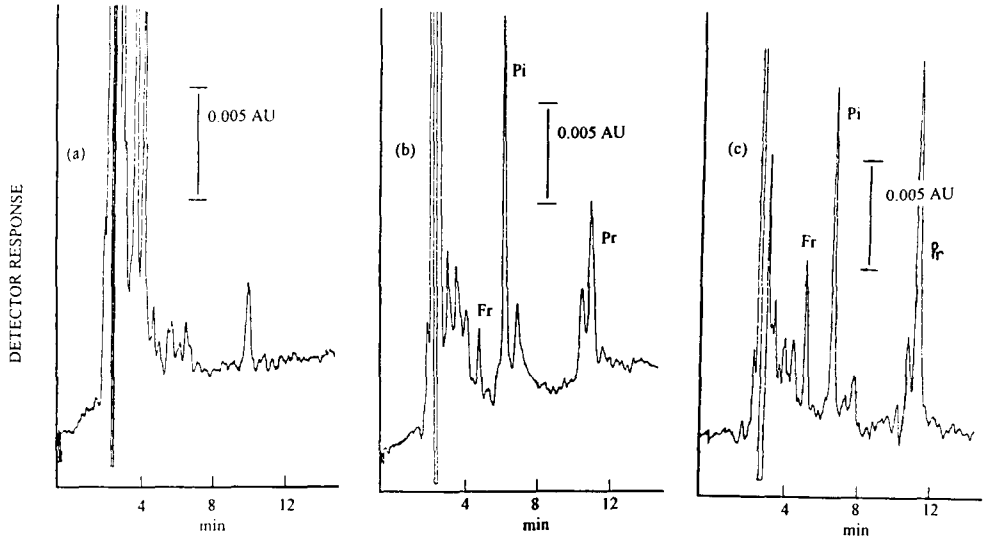


Fig. 4. Chromatograms showing (a) blank plasma, (b) the lowest concentration and (c) the highest concentration following extraction by column-switching. Compounds were separated on a Hypersil C18/SCX column using a mobile phase of 100 mM sodium acetate (pH 4)–acetonitrile (67:33 v/v) delivered at a flow rate of 1.0 ml min^{-1}

References

[1] D.G. Shand, New Engl. J. Med., 293 (1975) 280.
[2] A. Mitrani, M. Oettinger, E.G. Alunader, M. Sharf and A. Klein, Br. J. Obstet. Gynacol., 82 (1975) 651.
[3] K.L. Granville-Grossman and P. Turner, Lancet, 1 (1966) 788.
[4] H.S. Frazier and H. Yager, New Engl. J. Med., 288 (1973) 246.
[5] H.S. Frazier and H. Yager, New Engl. J. Med., 288 (1973) 455.
[6] M.P. Quaglio, A.M. Bellini, L. Minozzi, G. Frisina and F. Testoni, J. Pharm. Sci., 82 (1993) 87–90.
[7] H. Brzezinka, I. Bahr and H. Budzikiewicz, Biol. Mass Spectrom., 22 (1993) 462–464.
[8] B.R. Simmons and J.T. Stewart, J. Liq. Chromatogr., 17 (1994) 2675–2690.
[9] P. Hubert, P. Chiap, M. Moors, B. Bourguignon, D.L. Massart and J. Crommen, J. Chromatogr., 665 (1994) 87–99.
[10] F.F.T. Verves, H.G. Schaefer, J.T. Lefevre, L.M. Lopez and H. Derendorf, J. Pharm. Biomed. Anal., 8 (1990) 535–539.
[11] E. Bonet-Domingo, M.J. Medina-Hernandez, G. Ramis-Ramos and M.C. Garcia-Alvarez-Coque, J. Chromatogr., Biomed. Appl., 120 (1992) 189–194.
[12] T.B. Vree, M. Van-den-Biggelaar-Martea and C.P.W.G. Verway-van-Wissen, J. Chromatogr., Biomed Appl., 655 (1994) 53–62.
[13] H.W. Hagedorn and R. Schulz, J. Anal. Toxicol., 16 (1992) 194–198.
[14] L.J.C. Line-Love and J.J. Fett, J. Pharm. Biomed. Anal., 9 (1991) 323–333.
[15] B. Lindström, J. Chromatogr., 100 (1974) 189–191.
[16] A.L.M. Kerremans, Y. Tan, C.A.M. Van-Ginneken and F.W.J. Gribnau, J. Chromatogr., Biomed. Appl., 229 (1982) 129–139.
[17] M. Walshe, M.T. Kelly, M.R. Smyth and H. Ritchie, J. Chromatogr., 708 (1995) 31–40.