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Rapid and efficient purification of cimetropium bromide and mifentidine drug metabolite mixtures derived from microsomal incubates for analysis by mass spectrometry

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ABSTRACT

A comparative study of the use of organic solvent extraction *versus* Sep-Pak C_{18} cartridges in the recovery and analysis of phase I (unconjugated) drug metabolites using mass spectrometry is presented. Standard mixtures of putative metabolites of the anticholinergic drug cimetropium bromide and the H_2-antagonist mifentidine were purified from inactivated liver microsomal preparations using both methods, and subsequently the recovery of each compound was quantitated. In general, the percentage recovery and degree of purification were greater when using Sep-Pak C_{18} cartridges compared with organic solvent extraction. Even more efficient recovery was achieved when zinc sulphate precipitation of proteins in the liver microsomal mixtures was carried out prior to analysis. Also, the HPLC-grade solvents used in this study contained a variety of ultraviolet-inactive, hydrophobic components. This leads to problems of suppression in fast atom bombardment mass spectrometric analysis. Using Sep-Pak C_{18} cartridges directly prior to analysis by fast atom bombardment with single or tandem mass spectrometry leads to far superior mass spectral results compared with organic solvent extraction.

INTRODUCTION

Recently mass spectrometry (MS), and in particular tandem mass spectrometry (MS–MS) [1], has been increasingly used in drug metabolism studies [2–6]. MS has been utilized in the characterization and quantitation of metabolites found in complex biological matrices derived from both *in vitro* sources, *e.g.*, microsomal incubates [7,8], and *in vivo* sources, *e.g.* urine, blood, bile and faeces [5,9,10]. However, it is necessary to obtain clean samples free of cations, hydrocarbons and plasticizers, prior to analysis by MS. This is particularly relevant when fast atom bombardment mass spectrometry (FAB-MS) [11–13] is the primary ionization technique. The presence of hydrophobic contaminants leads to the well described phenomenon of suppression [14,15], and

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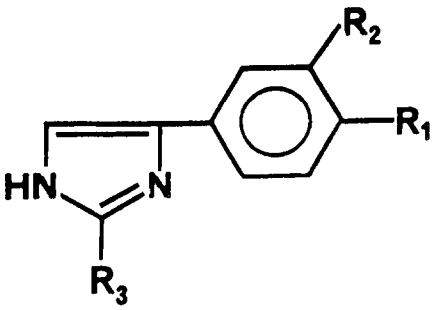
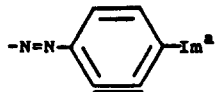
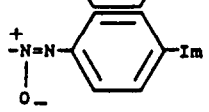
the presence of cations leads to multicationization of the sample, which effectively dilutes the abundance of the molecular ion [12].

Typically the recovery of xenobiotic compounds from biological mixtures is a multi-step process. In drug metabolism, solvents of varying polarity (*i.e.* diethyl ether, chloroform, acetonitrile) are commonly used for the extraction of phase I (unconjugated) metabolites [16,17], whereas Sep-Pak C_{18} reversed-phase cartridges are used for the extraction of phase II (conjugated) metabolites [18]. Extraction mixtures are usually analysed by conventional analytical methods, such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and thin-layer chromatography (TLC). However, identification and structural characterization of individual metabolities in such mixtures are hampered by the availability of only small amounts of metabolites (often in only nanomolar concentrations) or metabolites lacking UV absorption. Furthermore, the conventional analytical methods used are time-consuming and tedious.

To alleviate the problems of sample impurities,

(a)

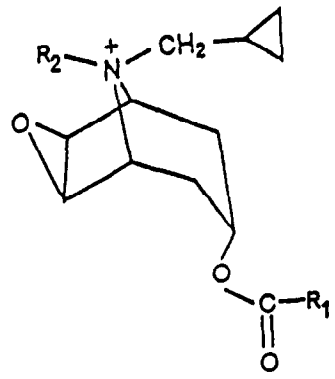


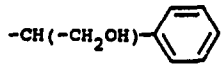
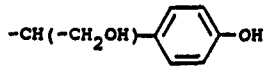
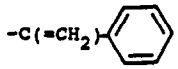
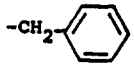
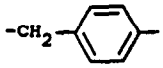
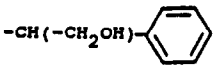
R_1	R_2	R_3	Compound
$-NH-CH=N-CH(CH_3)_2$	H	H	Mifentidine (I)
$-NH-C(=O)-NH-CH(CH_3)_2$	H	H	(II)
$-NH-CHO$	H	H	(III)
$-NH_2$	H	H	(IV)
$-NO_2$	H	H	(V)
$-NO$	H	H	(VI)
$-NH_2$	OH	H	(VII)
$-NH_2$	H	OH	(VIII)
	H	H	(IX)
	H	H	(X)

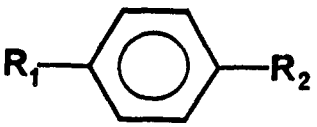
[a] = Imidazole

Fig. 1.

(b)



a) R_1	R_2	Compound
	CH_3	Cimetropium bromide (XI)
	CH_3	(XII)
	CH_3	(XIII)
	CH_3	(XIV)
	CH_3	(XV)
	H	(XVI)



b) R_1	R_2	Compound
$-CH(-CH_2OH)-COOH$	H	(XVII)
$-C(=CH_2)-COOH$	H	(XVIII)
$-CH_2-COOH$	H	(XIX)
$-CH_2-COOH$	OH	(XX)

Fig. 1. (a) Structures of mifentidine (I) and its synthetic analogues and/or metabolites (II–X). (b) Structures of cimetropium bromide (XI) and its synthetic analogues and/or metabolites (XII–XX).

as well as minimising sample loss prior to analysis by MS, numerous approaches have been adopted. Several groups have used column-switching HPLC techniques [19–22]. However, most other workers have used or recommended conventional methods such as Sep-Pak cartridges [23,24] or organic solvent extraction [25,26].

In this work we investigated the microsomal incubations of the H_2-antagonist, mifentidine (I) [7,27] and the anti-muscarinic drug cimetropium bromide (XI) [28] (see Fig. 1a and b, respectively). The aim of this study was to compare the use of Sep-Pak C_{18} cartridges with various organic solvent extractions. This should allow the determination of (i) the efficiency of each method in recovering metabolites from a microsomal incubate, and (ii) the "purity" of each metabolic mixture for analysis directly by FAB-MS and FAB-MS–MS.

EXPERIMENTAL

Chemicals

Mifentidine (I), cimetropium bromide (XI) and all synthetic standards were supplied by the Institute De Angeli (Milan, Italy). All other materials were obtained from BDH, FSA Laboratory Supplies and Boehringer Mannheim (UK). Sep-Pak C_{18} cartridges were obtained from Waters Assoc. (Watford, UK). All the FAB matrices were purchased from Aldrich (UK), and used without further purification.

Animals and microsomal incubations

Hepatic microsomes were prepared by the $CaCl_2$ precipitation method described by Lam *et al.* [29] from male albino Dunkin–Hartley guinea-pigs (400–600 g), Wistar rats (200–300 g), albino mice (30–40 g) and Syrian golden hamsters (80–100 g) fed a standard diet *ad libitum*.

The substrates mifentidine (I) and cimetropium bromide (XI) (4 μmol per flask) were each dissolved in 0.5 ml of water, and cofactors [NADP (disodium salt), β-nicotinamide-adenine dinucleotide phosphate, glucose-6-phosphate (disodium salt), glucose-6-dehydrogenase, $MgCl_2$ in 2 ml of phosphate buffer (0.2 m*M*, pH 7.4)] were added. The liver microsomal preparation (equivalent to 0.5 g of liver resuspended in 1 ml of phosphate buffer) was added and the contents were all incubated for 20 min in the case of I and 30 min for XI at 37°C in 25-ml Erlenmeyer flask, as described previously [30].

Preparation of standard calibration curves

Calibration curves for mifentidine (I), cimetropium bromide (XI) and their potential metabolites were obtained by using 0.1, 0.2, 0.3, 0.4, 0.5 μmol aliquots plus the appropriate internal standards [*i.e.* 4-aminoacetophenone (0.5 μmol of 4-AAP) for compounds I–X; and either benzyl alcohol (0.2 μmol) or glycopyrolate (250 μmol) for compounds XI–XX] and analysed by HPLC. The calibration curves were obtained by plotting the peak-area ratio (PAR) of sample to standard.

Solvent extraction method

The percentage recovery of I and XI and their potential metabolites was obtained by using cofactor solutions with inactivated liver microsomes (boiled for 15 min). Then 0.2 μmol of each compound was added to the microsomal mixture, plus 0.5 μmol of 4-AAP for I and either 250 μmol of glycopyrolate or 0.2 μmol of benzylalcohol for XI. Next, 3 ml of solvents (*i.e.* acetonitrile, diethyl ether, ethyl acetate) were added to each flask. The contents of the flasks were transferred to screw-capped glass tubes containing NaCl (1 g). In the case of XI and its potential metabolites, 40% (w/v) heptanesulphonic acid was also added at pH 7.9 to each tube containing solvent. The tubes were capped, "whirl-mixed", and placed on a bench rocking device for 1 h. The tubes were then centrifuged at 3000 *g* for 10 min, and the organic phase was removed and transferred to clean tubes using pasteur pipettes. The remaining aqueous phases were extracted twice more with 3-ml aliquots of solvents as described above. The combined organic phases were evaporated to dryness under nitrogen gas using a water-bath at 40°C. The concentrated extracts were redissolved in 100 μl of methanol and analysed by HPLC. The solvent extraction method was repeated three times for each solvent, and the per-

centage recovery was approximately the same (±3%) on each occasion.

In the case of XI, acetonitrile was used for the extraction of all metabolites from liver microsomal incubates; after the second extraction, the pH of aqueous phase was lowered to 3.7 by the addition of 47.2 μl of concentrated HCl, and the phase extracted with a further two 5-ml volumes of acetonitrile as before.

Sep-Pak C_{18} extraction method

The contents of the Erlenmeyer flasks (described above) were transferred to 12-ml centrifuge tubes, and 1 ml of 1% $ZnSO_4$ (w/v) was added. The tubes were centrifuged at 3000 *g* for 15 min. The supernatants were loaded onto a prewashed C_{18} Sep-Pak column, washed with 0.5 ml of water and then eluted with 5 ml of methanol. The methanol was removed under nitrogen at 37°C. The residue was redissolved in 100 μl of methanol for analysis by HPLC and MS.

HPLC system

Ion-paired reversed-phase chromatography was used for both I [27] and XI [28]. For I and its metabolites, chromatography was performed on a 10 μm Bondapak ODS-2 column (25 cm × 0.46 cm I.D.) using an Altex 110A pump and a Beckman Model 110B pump connected to a Beckman mixing chamber. The aqueous component (A) of the mobile phase was 10 m*M* NaH_2PO_4 containing 8 m*M* heptanesulphonic acid (sodium salt) plus 30 m*M* triethylamine (overall pH 3.4); the organic component (B) was 100% acetonitrile. The composition of the mobile phase at $t = 0$ was 90% A; at $t = 8$ min, 80% A; at $t = 13$ min, 80% A; at $t = 20$ min, 90% A; and at $t = 30$ min, 90% A. The flow-rate was 1 ml/min.

For XI and its metabolites chromatography was performed on a 10 μm Spherisorb ODS-2 column (25 cm × 0.46 cm I.D.) using isocratic elution at a flow-rate of 1 ml/min via an Altex 110A pump. The most suitable mobile phase was methanol–acetonitrile–water [either 20:20:55 (v/v) or 5:10:55 (v/v)], containing heptanesulphonic acid (sodium salt) (either 0.035 or 0.02 *M*) plus triethylamine (0.1 *M*) with a pH of 3.7.

Mass spectrometry

All mass spectra were obtained on a VG 70-SEQ instrument of EBQ_1Q_2 configuration, where E is an electrostatic analyser, B is the magnet, Q_1 is an rf-only quadrupole collision cell and Q_2 is a mass filter quadrupole. EB and Q_2 correspond to mass spectrometers 1 (MS_1) and 2 (MS_2), respectively. All synthetic standards and microsomal incubate mixtures were ionized by positive-ion FAB-MS. Xenon atoms from a Model B11N saddle-field fast-atom gun (Ion Tech, Teddington, UK) were used as the primary ionizing beam and collided with the sample at 8.5 keV. The secondary ions produced by the fast xenon atoms were accelerated out of the source region to an energy of 8 keV, and the magnet scanned at 5 s per decade over the mass range m/z 1350–50.

To obtain constant neutral loss (CNL) spectra, both mass spectrometers MS_1 and MS_2 were adjusted to transmit only ions of mass m_1^+ and m_2^+, respectively, such that $(m_1 - m_2) = m_c$, where m_c is the chosen mass of the neutral fragment (in this case 54 daltons) [1]. The two mass spectrometers MS_1 and MS_2 were then scanned simultaneously such that the mass difference (m_c) was maintained throughout the scan. All resulting m_2^+ daughter ions detected could arise only from molecular ions of mass difference m_c, and the resulting CNL spectrum contained all the molecular ions that gave rise to a CNL of m_c. Specifically, a scan speed of 30 s was selected over the mass range 450–50 daltons. The collision gas cell conditions were maintained at a collision gas pressure (argon) of 10^{-7} mbar and a collision energy of 10 eV. All scans were obtained in the multi-channel analysis (MCA) mode.

Sample preparation for mass spectrometry

Both the synthetic standards and the microsomal mixtures (post Sep-Pak) were dissolved in methanol, and 1 μl of this solution was mixed with 1.5 μl of the FAB matrix, 3-nitrobenzylalcohol, on a stainless-steel probe tip and subsequently inserted into the mass spectrometer.

RESULTS AND DISCUSSION

The usual procedure for determining the optimum extraction conditions for the recovery of complex biological mixtures in drug metabolism research is to extract first with diethyl ether. The advantage of using this solvent is the ease of removal after extraction using low temperatures (*e.g.* 37°C) or nitrogen gas. This is particularly imporant when trying to isolate and characterize thermally labile compounds, such as N-oxides and hydroxylamine metabolites. Subsequent extractions with solvents of increasing polarity on inactivated microsomes containing metabolite standard mixtures are then performed to ascertain percentage recoveries. Ultimately, a suitable solvent or combination of solvents is decided on for use with the "live" microsomal incubation mixtures [31]. However, since solvent extractions are controlled by a variety of different parameters, including partition coefficients of metabolites and solvent miscibilities, the efficiencies of extraction for two structurally similar compounds into the same extractive solvent can be dramatically different.

The variable recovery of metabolite standards using a number of solvents of increasing polarity (*i.e.* diethyl ether, $\varepsilon_{20} = 4.3$; chloroform, $\varepsilon_{20} = 4.8$; ethyl acetate, $\varepsilon_{20} = 6.0$; dichloromethane, $\varepsilon_{20} = 9.1$; and acetonitrile, $\varepsilon_{20} = 38.8$) is highlighted in Tables I and II. Table I shows the recovery of mifentidine (I) and its synthetic analogues II–X from an inactivated microsomal incubate after a single solvent extraction for a variety of solvents, plus the recovery of the same compounds using a Sep-Pak C_{18} cartridge. Table II documents a similar set of results for the recovery of cimetropium bromide (XI) and its synthetic analogues XII–XX, also from an inactivated microsomal incubate, but in this case repeated extractions with the same solvent were carried out.

In both cases (Table I and II) the percentage recovery of specific compounds varies greatly from solvent to solvent. Also, compounds of comparable polarity (*e.g.* VII and VIII in diethyl ether) are not recovered to any degree of similarity using the same extraction solvent.

The results shown in Tables I and II clearly highlight the drawbacks of using the solvent extraction method to isolate a complex mixture of drug metabolites from a microsomal incubate.

TABLE I

PERCENTAGE RECOVERY OF MIFENTIDINE (I) AND ITS SYNTHETIC AND/OR METABOLITE ANALOGUES (II–X) FROM A MICROSOMAL INCUBATION MIXTURE

Comparison of various single solvent extractions with Sep-Pak C_{18} cartridge.

Compound	Recovery (%)					
	CH_3CN	CH_2Cl_2	CH_3COOEt	$CHCl_3$	Et_2O	Sep-Pak C_{18}
I	59	2	7	2	N.D.[a]	85
II	85	34	68	26	44	86
III	37	3	45	N.D.	7	100
IV	55	8	51	4	7	100
V	54	73	82	87	70	90
VI	84	55	99	77	52	99
VII	12	2	11	N.D.	4	66
VIII	21	N.D.	6	N.D.	54	77
IX	38	20	72	15	23	80
X	28	8	56	100	N.D.	87

[a] N.D. = not detected.

TABLE II

PERCENTAGE RECOVERY OF CIMETROPIUM BROMIDE (XI) AND ITS SYNTHETIC AND/OR METABOLITE ANALOGUES (XII–XX) FROM A MICROSOMAL INCUBATION MIXTURE

Comparison of various single solvent extractions with Sep-Pak C_{18} cartridge.

Compound	Recovery (%)					
	CH_3CN[a]	CH_2Cl_2[b]	CH_3COOEt[b]	$CHCl_3$[b]	Et_2O[b]	Sep-Pak C_{18}[c]
XI	100	62	32	75	54	100
XII	96	N.D.[d]	2	N.D.	N.D.	81
XIII	80	71	16	43	N.D.	76
XIV	100	100	10	80	N.D.	100
XV	100	14	12	10	N.D.	62
XVI	73	39	63	67	61	78
XVII	100	N.D.	60	5	17	66
XVII	93	79	100	97	100	98
XIX	100	66	100	98	100	100
XX	100	6	100	3	82	100

[a] Extraction with two 5-ml volumes at pH 7.4 plus 40% (w/v) heptanesulphonic acid plus NaCl (1 g), following extraction with two 5-ml volumes at pH 3.7.

[b] Two 5-ml volumes at pH 3.7.

[c] Extration at pH 7.4.

[d] N.D. = not detected.

Firstly, it is difficult *a priori* to predict which solvent will be the most efficient in extracting the maximum number of metabolites at the greatest possible extraction efficiencies. Hence a time-consuming set of experiments to determine which solvent is best suited for a particular mixture of metabolites must be undertaken. Secondly, the polarity of the solvent will determine that only metabolites of similar polarity will be extracted efficiently, whereas metabolites that are either much more polar than the extraction solvent (*e.g.* XI–XV in diethyl ether) or much less polar (*e.g.* V in acetonitrile) will not be recovered in reasonable yields.

The use of zinc sulphate to precipitate out the microsomal protein fraction, followed by loading the supernatant onto a Sep-Pak C_{18} cartridge, results in a uniformly high percentage recovery. The compounds loaded and recovered in this work range from the quaternary ammonium salts of cimetropium bromide (XI–XVI) and the aromatic carboxylic acids (XVII–XX) to the mifentidine compounds, which possess a wide range of functional groups of widely differing polarity (I–X). The percentage recovery of the synthetic standards from inactivated microsomal incubates is always greater than 60% and in many cases exceeds 90%, as shown in Tables I and II.

Invariably after isolation of the crude mixture of metabolites it is necessary to separate the mixture into pure compounds prior to spectroscopic analysis. The usual method of choice for drug metabolite mixtures is HPLC, in conjuction with UV studies to try to ascertain structural information about the metabolites. However, it is not uncommon for both *in vivo* and microsomal incubation mixtures to contain very low concentrations of metabolites, hence any further purification leads to even greater losses prior to spectroscopic analysis. Fig. 2 details the difference in using CH_3CN to extract a mixture of mifentidine (I) plus three of its analogues (III, IV, and VIII) from an inactivated microsomal incubate *versus* using the combined zinc sulphate precipitation

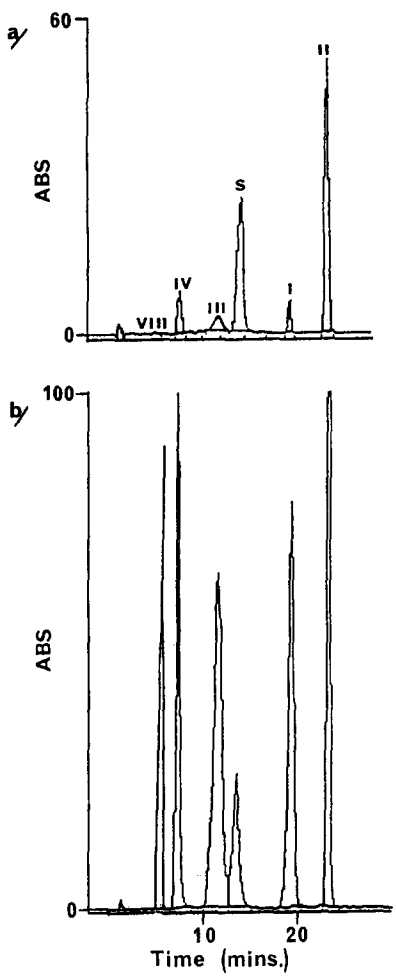


Fig. 2. HPLC of a mixture of mifentidine (I) plus four metabolite standards (II, III, IV and VIII) and the internal standard (S) after (a) acetonitrile extract of an inactivated microsomal incubate and (b) zinc sulphate precipitation and Sep-Pak C_{18} cartridge treatment of an identical inactivated microsomal incubate.

and Sep-Pak C_{18} cartridge method (Fig. 2b). It can be seen from Fig. 2a that, in the chromatogram of the CH_3CN extract, III and VIII are barely detectable, whereas using the Sep-Pak method all five compounds are clearly visible above the background.

One of the most powerful techniques used to ascertain the presence of metabolites in complex mixtures is MS. In particular, the advent of soft ionization techniques, such as FAB-MS, has revolutionized our ability to detect and characterize a wide range of polar, thermally labile compounds. Furthermore, using FAB-MS to screen for possible metabolites, in conjunction with FAB-MS-MS to structurally characterize individual metabolites [5–7], does not necessitate the purification of individual compounds by HPLC.

Recently we investigated the *"in vitro"* metabolism of mifentidine (I) using guinea-pig hepatic microsomes [7]. Initial screening of the microsomal incubate after solvent extraction with CH_3CN revealed no informative ions in the positive ion FAB mass spectrum (Fig. 3a). The only prominent ions observed corresponded either to matrix and contaminant ions (*e.g.* *m/z* 166, 176, 184, 192) or the substrate mifentidine (*m/z* 229). However, taking an identical microsomal incubate and subjecting it to zinc sulphate precipitation followed by loading onto a Sep-Pak C_{18} cartridge and subjecting it to FAB-MS revealed prominent ions at *m/z* 160, 188, 229 and 245 corresponding to IV, III, substrate I, and II, respectively.

A further example serves to highlight the problem of using the solvent extraction method immediately prior to MS analysis. Various hepatic microsomal incubates of cimetropium bromide (XI) were subjected to CNL scanning in order to rapidly detect new metabolites of this drug [32]. Initial studies used metabolite mixtures that had been extracted with acetonitrile. As shown in Table II, the efficiency of extraction by acetonitrile for XI and its potential metabolites is high. However, when the mixture was subjected to tandem MS CNL scanning, no ions could be detected other than substrate, matrix and contaminant ions (Fig. 4a). However, as with mifentidine, use of the Sep-Pak method enabled us to rapidly identify the presence of at least four metabolites, at *m/z* 210, 328, 374 and 388, plus substrate at *m/z* 358 [32], and this is detailed in Fig. 4b.

In both the mifentidine and cimetropium bromide cases the solvent extraction method used HPLC-grade acetonitrile. However, although such solvents are guaranteed to be free of UV-absorbing materials, they tend to contain a sizeable amount of hydrophobic contaminants, such as aliphatic compounds. As is now well docu-

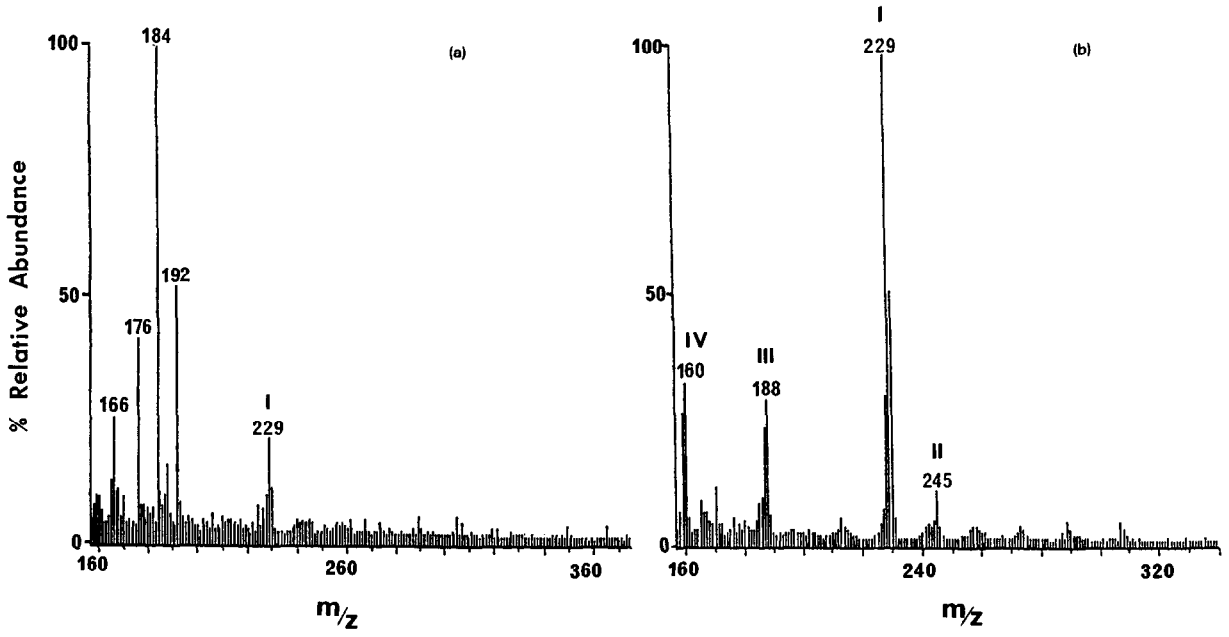


Fig. 3. Positive-ion FAB-MS spectrum of a microsomal incubation of mifentidine (I) after (a) acetonitrile extraction and (b) zinc sulphate precipitation in conjuction with Sep-Pak C_{18} cartridge purification. The ions at m/z 160, 188, 229, and 245 correspond to IV, III, I and II respectively.

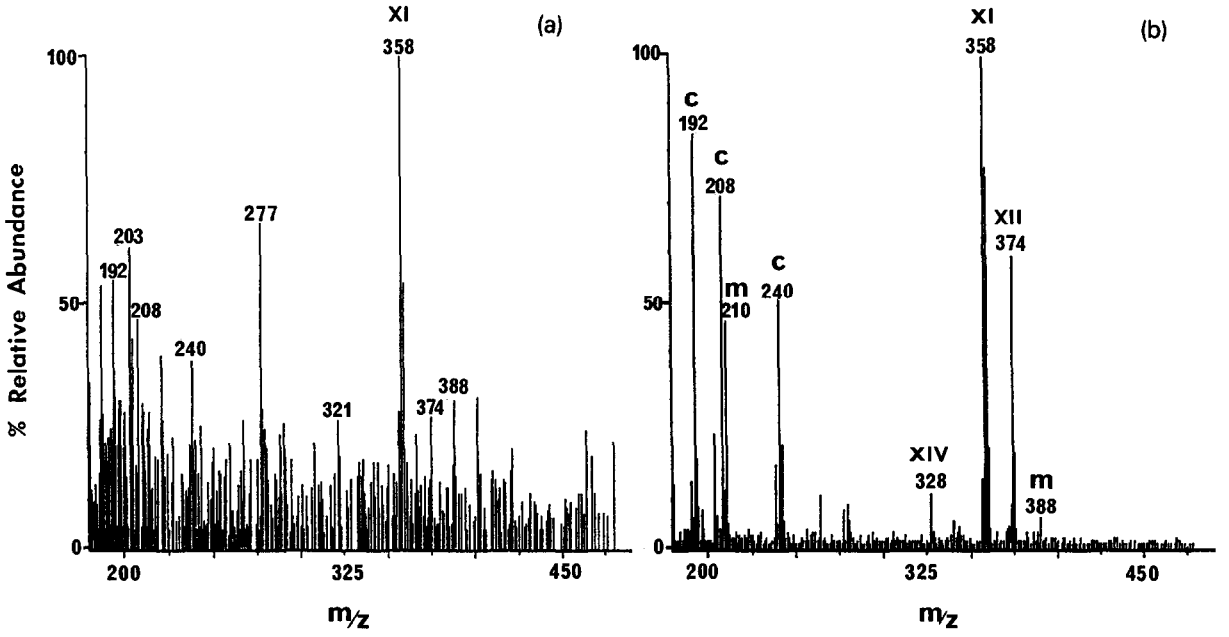


Fig. 4. Constant neutral loss spectrum of a microsomal incubation of cimetropium bromide (XI) after (a) acetonitrile extraction and (b) zinc sulphate precipitation in conjunction with Sep-Pak C_{18} cartridge purification. The ions at m/z 328, 358 and 374 correspond to XIV, XI and XII, respectively. c = ions observed in "control" spectra; m = unknown metabolite(s).

mented [14,15] such compounds readily suppress signal ion abundance of polar analyte molecules, and lead to no signal being observed. However, when the Sep-Pak method is used, no such compounds are introduced into the microsomal incubate, and any very hydrophobic contaminants can be selectively eluted off the Sep-Pak C_{18} cartridge after removal of the polar analytes.

CONCLUSION

In this work we demonstrate that the combined use of zinc sulphate precipitation in conjunction with Sep-Pak C_{18} cartridges is far superior to simple solvent extraction to recover drug metabolites from a microsomal incubation mixture.

The samples obtained after Sep-Pak C_{18} cartridge treatment are clean and do not require further purification prior to MS analysis. Furthermore, the overall recovery using the Sep-Pak method results in consistently high recovery levels of metabolite standards from a microsomal incubate, whereas the use of a conventional organic solvent generally does not adequately extract efficiently all of the metabolite standards present.

In summary, the use of the Sep-Pak C_{18} method has the following advantages over the solvent extraction method: (1) a high percentage of all metabolites are recovered in a single step; (2) the method is cheap and very fast; (3) water and methanol are the only solvents required, and hence it is possible to remove the possibility of introducing major hydrophobic contamination into samples prior to MS analysis.

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