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A generic approach for the determination of residues of alkylating agents in active pharmaceutical ingredients by *in situ* derivatization–headspace–gas chromatography–mass spectrometry

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Abstract

A simple, reliable and fast procedure for the simultaneous determination of residues of some common alkylating agents (AAs), such as mesylates, besylates, tosylates and sulfates, employed in drug synthesis, has been developed by *in situ* derivatization–headspace–gas chromatography–mass spectrometry.

Pentafluorothiophenol is used as a derivatizing agent in different water/dimethyl sulfoxide ratios. Compared to former analytical procedures, this approach returns improvements in analysis time, selectivity, analyte stability and method sensitivity (LOD = 0.11 $\mu g\,g^{-1}$ for methyl tosylate). The method exhibits low matrix dependence, excellent accuracy, precision (R.S.D. = 2.8–10% range at 1 $\mu g\,g^{-1}$) and robustness through the use of deuterated internal standards. Knowledge of the synthetic route allows a targeted approach to the determination of specific AAs since the procedure does not distinguish between acid species. The procedure was successfully applied to different pharmaceutical matrixes, and is particularly suitable for routine analysis with high sample throughput.

Keywords: Alkylating agents; Derivatization; Headspace; Capillary gas chromatography; Mass spectrometry; Pharmaceutical products

1. Introduction

The synthesis of pharmaceutical products often involves the use of reactive reagents for the formation of intermediates and active pharmaceutical ingredients (APIs). Low-levels of reagents or by-products may therefore be present in the final API as impurities. Such chemically reactive impurities may have unwanted toxicities, including genotoxicity and carcinogenicity, and hence the potential impact on product quality and risk profile requires consideration and management [1]. The pharmaceutical industry recognizes their obligation to control genotoxic impurities to appropriately safe levels in pharmaceutical products destined for human or animal use [2].

Alkylating agents (AAs) are a class of potentially genotoxic impurities that require a control strategy in pharmaceutical process development, and may require control in API or during manufacture. AAs may be used as reagents or can be produced when strong acids (e.g. H_2SO_4) react with alcohols (used as solvents or present as impurities) to form the corresponding ester [3].

AAs are reactive compounds that have a broad range of physico-chemical properties, and specific analytical methodologies are usually required for the determination of each species. Development and robustness issues are commonplace. Different approaches have been proposed to determine AAs in API that involve biosensors [4–6], isotachophoresis [7], gas chromatog-

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raphy [8–12] and liquid chromatography [13,14]. Analytical methodologies presently available for AA determinations have significant constraints. They are time-consuming, exhibit low sensitivity, and suffer from interferences from the matrix or from the derivatization agents used [13,15,16]. The methods are typically limited by the solvent used during the sample preparation step, requiring high solubility of the matrix, for example in water, and thus limiting their application for the broad range of matrixes encountered in pharmaceutical development [8,10]. Direct injection as well as direct solid phase microextraction (SPME) of complex API solutions can raise contamination issues in the GC injection port due to the presence of non-volatile components. This contamination may result in repeatability and reproducibility problems that can prevent successful method validation [10,12]. Derivatization agents have been employed to produce more GC amenable analogues of the target analytes. The use of thiocyanate as derivatizing agent has been reported for alkyl methanesulfonate determination [8], but this approach demonstrates severe drawbacks for example high aqueous solubility requirements, multiple product formation prone to isomerisation and low mass selectivity.

A method for the determination of mesylates, besylates, tosylates and sulfates that takes advantage of their reactivity in forming derivatives is presented. The resultant products are more volatile, stable, less polar and easier to extract than the parent compounds. The derivatives are therefore more amenable to GC analysis, and also contain groups that significantly enhance detection. For a method to be generic, the solvents used should be versatile in dissolving pharmaceutical compounds. A range of polar, inert, aprotic and high boiling solvents is employed successfully for the determination of volatile organic impurities (VOIs) in pharmaceuticals [17]. This general description of a good solvent for pharmaceuticals was therefore used to identify possible candidates. In addition the optimum solvent or solvents would have to enhance response for the derivatives by facilitating the derivatization reaction, and yielding a favourable partition of the derivatives during headspace sampling.

The aim of this work was to develop a generic, rapid, selective, sensitive, high throughput and robust method for the determination of AA impurities in APIs at low-level concentration.

2. Experimental

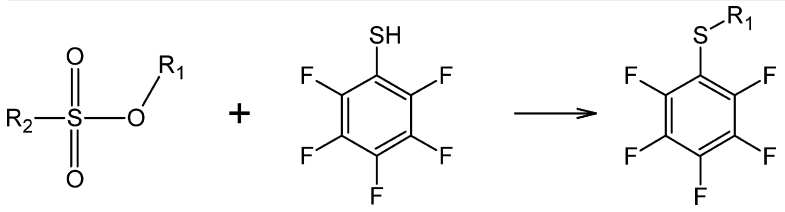
2.1. Chemicals and materials

The following reagents were obtained from Sigma–Aldrich (Steinheim, Germany) and used as received: dimethyl sulfate (DMeS, +99%), diethyl sulfate (DEtS, 98%), methyl methanesulfonate (MeMS, 99%), ethyl methanesulfonate (EtMS, 99%), methyl *p*-toluenesulfonate (MepTS, 98%), ethyl *p*-toluenesulfonate (EtpTS, 98%), methanol-d_4 (99.8%), ethanol-d_6 (anhydrous, 99.5%), isopropanol-d_8 (+99%), methane sulfonic acid (99.5+%), *p*-toluene sulfonic acid monohydrate (98.5+%), hexamethyl phosphoramide (HMPA, 99%), 2,5-dichlorobenzenethiol (DCBP, 98%), pentachlorothiophenol (PCTP, 96%), 2,3,5,6-tetrafluorothiophenol (TFTP, 97%) and pentafluorothiophenol (PFTP, 97%). Ethyl benzenesulfonate (EtBS) and di-isopropyl sulfate (D*i*PrS) were supplied by TCI-EP (Tokyo, Japan). Isopropyl *p*-toluenesulfonate (*i*PrpTS) and isopropyl benzenesulfonate (*i*PrBS) were prepared by the Chemical R&D Department at Pfizer (Sandwich, UK) and high purity values (>98%) for both chemicals was ascertained by GC–MS and NMR. Acetonitrile (ACN, Chromasolv for HPLC gradient), dimethyl formamide (DMF), dimethyl sulfoxide (DMSO, >99.7%) were from Fluka Chemie GmbH (Buchs, Switzerland). *N*,*N*-dimethyl acetamide (DMAC, HPLC grade) was from Rathburn Chemicals Ltd. (Walkerburn, Scotland), dichloromethane (DCM), water (Millipore, Milli-Q purified), sodium chloride (NaCl) and sodium hydroxide (NaOH, analytical reagent grade) from Fisher Scientific UK (Loughborough, England). For the internal standard preparation, and in addition to the free acids and deuterated alcohols listed: isopropyl methanesulfonate (*i*PrMS, 99%) and methyl benzenesulfonate (MBS, 99%) were from Acros Organics (Geel, Belgium). Sulfuric acid (analytical grade 98%) and sodium sulfate anhydrous (>99%) were purchased from BDH VWR Int. Ltd. (Poole, England). The SPE clean-up procedure employed Strata C_8 cartridges (50 μm, 100 mg/1.0 mL and endcapped) from Phenomenex (Torrance, CA, USA). AAs stock and working standard solutions were prepared in ACN and stored at 4 °C. Experiments were carried out in an extracted, clean environment to avoid exposure and contamination. After analysis, vial samples were discharged for incineration, avoiding any further manipulation.

2.2. Internal standard synthesis

Nine deuterated AAs selected to control and provide insight into the derivatization, extraction and detection variables were synthesized (i.e. DMeS-d_3, DEtS-d_5, D*i*PrS-d_7, MeMS-d_3, EtMS-d_5, *i*PrMS-d_7, MepTS-d_3, EtpTS-d_5 and *i*PrpTS-d_7). Deuterated-tosylates were used as internal standards for besylate determinations (i.e alkyl benzenesulfonates), due to the similarity of their physico-chemical properties. For each deuterated internal standard, 100 μL of the appropriate deuterated alcohol (i.e. methanol-d_4, ethanol-d_6 and isopropanol-d_8) were mixed with 10 μL of the corresponding acid (i.e. sulfuric acid, methanesulfonic acid and *p*-toluenesulfonic acid), in separate 5.0 mL microreaction vessels (Supelco, Bellafonte, PA, USA), and refluxed in a thermostatic block system (Pierce, Chicago, IL, USA) at 100 °C. After 2 h reaction, vessels were cooled to room temperature. 1.5 mL of water (saturated with NaCl) and 1.5 mL of DCM were added to the vial. Liquid–liquid extraction (LLE) was performed three times and the recovered organic phase was passed through a 0.5 g column of anhydrous Na_2SO_4. High purity values (>98%) were obtained as monitored by GC–MS. AA deuterated stock and working standard solutions were diluted to 20 mL using acetonitrile, and stored at 4 °C in a sealed vessel. Appropriate mixtures of AA deuterated working solution concentrations (e.g. DMS-d_3, DES-d_5 and D*i*PS-d_7) were prepared. Concentration ranges were targeted to obtain a similar MS response to 1.0 μg g^{-1} of their corresponding AAs. No interferences were detected using this approach (i.e. the blank level was not affected).

Table 1
Derivatization reaction, retention time and selected ions for the determination of alkylating agents as their pentafluorothiophenol derivatives



Compound	Abbreviation	R_1 (analyte)	R_1 (IS)	Retention time (min)		Diagnostic ions (m/z^+)
				Analyte	IS	Analyte, IS
Mesylates ($R_2 = –CH_3$)						
Methyl methanesulfonate	MeMS	$–CH_3$	$–CD_3$	4.34	4.33	214, 217
Ethyl methanesulfonate	EtMS	$–CH_2CH_3$	$–CD_2CD_3$	4.45	4.44	228, 233
Isopropyl methanesulfonate	*i*PrMS	$–CH(CH_3)_2$	$–CD(CD_3)_2$	4.64	4.62	242, 249
Besylates ($R_2 = –C_6H_5$)						
Methyl benzenesulfonate	MeBS	$–CH_3$	$–CD_3$	4.34	4.33	214, 217
Ethyl benzenesulfonate	EtBS	$–CH_2CH_3$	$–CD_2CD_3$	4.45	4.44	228, 233
Isopropyl benzenesulfonate	*i*PrBS	$–CH(CH_3)_2$	$–CD(CD_3)_2$	4.64	4.62	242, 249
Tosylates ($R_2 = –C_6H_4CH_3$)						
Methyl *p*-toluenesulfonate	MepTS	$–CH_3$	$–CD_3$	4.34	4.33	214, 217
Ethyl *p*-toluenesulfonate	EtpTS	$–CH_2CH_3$	$–CD_2CD_3$	4.45	4.44	228, 233
Isopropyl *p*-toluenesulfonate	*i*PrpTS	$–CH(CH_3)_2$	$–CD(CD_3)_2$	4.64	4.62	242, 249
Sulfates ($R_2 = –OR_1$)						
Dimethyl sulfate	DMeS	$–CH_3$	$–CD_3$	4.34	4.33	214, 217
Diethyl sulfate	DEtS	$–CH_2CH_3$	$–CD_2CD_3$	4.45	4.44	228, 233
Diisopropyl sulfate	D*i*PrS	$–CH(CH_3)_2$	$–CD(CD_3)_2$	4.64	4.62	242, 249

2.3. Sample treatment

The sample (typically 5–50 mg of API or intermediate) was weighed into a 20 mL headspace (HS) vial and dissolved in 4.0 mL of a DMSO/water mixture (e.g. 1:1). A microliter amount of working and/or deuterated standard solutions (e.g. 10.0 μL) was spiked into the sample and the vial was sealed using an aluminium cap with a (PFTE/butyl rubber) septum (Agilent, Palo Alto, CA, USA). One hundred microliter of the derivatization agent solution (6.4 mg mL^{-1} in NaOH 1.0 M) was added through the septum using a microsyringe. Samples were shaken for 0.3 min and loaded into the HS sampler. Different organic solvents (ACN, DMSO, DMF, HMPA, DMAC)/water mixtures, as well as different concentration ratios were tested in order to select the most suitable solvent for the derivatization step. The reaction temperature (22–105 °C), and time (0–60 min) were optimized using experimental design. A surface of response was obtained in order to select the optimum conditions. The presence of alcohol (e.g. methanol, ethanol and *iso*-propanol), in the sample was tested at different concentration ranges (0.01, 0.1, 1.0 and 5.0% as a residual solvent) to evaluate their potential interference (selectivity) in the analytical approach.

Different pharmaceutical matrixes were tested, applying the analytical method developed and analysing alkyl (methyl, ethyl and/or isopropyl) tosylates, mesylates, besylates and dialkyl sulfates. For illustration, two samples from an intermediate API synthetic route were selected. Sample A is a tosylate salt exhibiting a high solubility in water while sample B is a less polar compound requiring an organic solvent to dissolve it. In both samples methyl, ethyl and isopropyl tosylates were potentially present at low concentration.

2.4. Headspace–GC–MS analysis

AA determinations were carried out using an Agilent system (Palo Alto, CA, USA) with HS injector (Model 7694), a GC (HP 6890N series), and a quadrupole MSD (Model 5973N). Optimum experimental conditions for the HS injector are: oven temperature 105 °C; sample loop 3 mL; transfer line and loop temperatures 140 °C; equilibration time 15 min; high speed agitation; GC cycle time 12 min; vial pressure 16 psi for 0.5 min; loop fill time 0.05 min; loop equilibration time 0.3 min; injection time 2.0 min. The valve and loop were of nickel and the transfer line was of Silcosteel®. The transfer line was inserted into the split–splitless GC injector.

The best column was a RH-624 (30 m × 0.32 mm i.d. × 1.8 μm from Capital Analytical Ltd., Leeds, UK). The optimum gas chromatographic conditions were as follows: initial oven temperature 60 °C (1 min) programmed to 250 °C at 50 °C min^{-1} and held at this final temperature for 1.2 min. Helium was used as carrier gas (constant flow 1.2 mL min^{-1}). The injector temperature was 220 °C in split mode (16.0 mL min^{-1}).

Chemstation D.01.02 software was used for data acquisition. The MS was operated with electron impact ionisation (70 eV, ionisation current 350 μA, source temperature 230 °C, transfer

line 250 °C), in selected ion monitoring mode (SIM) with a dwell time of 100 ms and a solvent delay of 4.1 min. For the measurement of AAs and their respective deuterated internal standard, the molecular ion of the derivative product was selected as a diagnostic fragment ion for retention times and monitored ions were used for quantitation (Table 1). AA calibration curves were constructed as a ratio between the AA standard areas to AA deuterated IS.

For real sample analyses, a standard addition calibration curve was developed. The linearity was evaluated from 0.25 to 50 $\mu g\, g^{-1}$ for each AA-. Detection (LOD) and quantification (LOQ) limits were calculated from low concentration values of the calibration curves, by considering the peak area corresponding from 3 (3σ) to 10 (10σ) times the signal-to-noise ratio of a procedural blank.

3. Results and discussion

Over the years, different analytical approaches have been developed to analyse AAs involving either LC or GC (unpublished methods). AAs are semi-volatile species and GC amenable, however, they are not suitable to be analysed directly. AAs can perform badly both in GC and LC analysis because of their high reactivity. In addition, we have found that AA determinations are highly matrix dependent. Although it is possible to carry out the analysis using both separation techniques, when a pharmaceutical sample is spiked at a low-level with the analyte, the recovery obtained is generally low, dramatically affecting the limit of detection (LOD). There are three crucial issues in trace level analysis of AAs, that influence the success of a determination: (a) matrix effects, (b) the selectivity of the pre-concentration step and (c) the selectivity of detection. A combination of these factors is the key to the development of a reliable trace analytical method.

In order to detect AAs at low concentration ($\mu g\, g^{-1}$) in pharmaceutical samples, it is mandatory to develop a highly selective clean-up step to separate the analytes from the bulk matrix. Matrix effects may also have an adverse impact on analytical data, they can introduce interfering substances into the background, and make results unreliable. Typically, suppression or enhancement of analyte response is accompanied by diminished precision and accuracy of subsequence measurements. Even when highly selective detection is applied (e.g. LC–MS–MS), this drawback is not overcome [18,19]. Traditional fractionation procedures are time consuming and are also matrix specific.

The ideal generic analytical approach is to isolate the analytes from the matrix prior to their separation in the chromatographic or MS dimension. Among all the analytical techniques suitable for the separation of AAs from the API matrix, static headspace (HS) sampling is by far the best choice [20]. This technique is suitable for a variety of organic/aqueous solvent mixtures, and is the most readily automated and validated sampling technique presently available [21–24]. Derivatization reactions are applied in GC to convert analytes into volatile and stable species suitable for GC and/or to improve detection. Although this approach is time consuming and can adversely affect the accuracy and precision of a determination, it is a powerful tool that can overcome many development limitations. AAs are common reagents employed in GC derivatization reactions and one can take advantage of their reactivity by transforming them into species suitable for sampling by HS. HS amenable derivatization can be even more advantageous because of the excellent matrix handling and selective extraction afforded by this technique. A reduction of intermolecular association is particularly important for analysis by gas chromatography and mass spectrometry, as it can facilitate the application of these techniques to compounds not directly amenable due to low volatility.

3.1. Derivatization

The analytical approach developed takes advantage of the chemical reactivity of the AAs to transform them into more volatile species suitable for HS extraction and GC analysis. Functionality, which would also enhance detection, was sought. Different derivatization agents were reviewed in order to determine which of them exhibited the best characteristics for HS–GC–MS analysis.

Aryl thiols (mercaptans) are amongst the strongest nucleophilic species, and have been successfully applied as derivatizing reagents to the determination of antineoplastic agents (i.e. busulfan or 1,4-butanediol dimethanesulfonate) by GC–MS [25–27] or GC-ECD [28–30]. A number of aryl thiols with halogenated groups, were considered as derivatization agents. The product sulfides are prepared by treatment of a primary or secondary alkylated agent with a thiolate anion ($R'S^-$). The reaction occurs by an S_N^2 mechanism (analogous to the Williamson synthesis of ethers) and product yields are usually high in these substitution reactions. Due to the low sulfide concentration produced as well as their high volatility, trialkylsulfonium salt by-products are avoided [31].

Several chlorothiophenols (DCBT and PCTP) and fluorothiophenols (TFTP and PFTP) were tested using HS–GC–MS. The derivatization reaction is in basic medium in order to generate the corresponding aryl-thiolate nucleophile. These nucleophiles are not volatile and are therefore ideal for HS sampling since no further clean-up step is required. Basic conditions also reduce the potential for formation of AAs in the presence of residual alcohols and strong acids.

Although the chlorinated compounds exhibited higher response in MS compared to the fluorinated compounds, their vapour pressure and partitioning into the headspace was found to be insufficient for HS sampling at usable concentrations. Moreover, the chlorinated derivatives had longer retention times compared to the fluorinated derivatives and co-elution of ethyl and isopropyl derivatives was noted. Alkyl-fluorothiophenol derivatives exhibited more suitable properties for HS–GC–MS. They partition well into the HS and the chosen GC column delivered a highly selective separation [32]. PFTP derivatives gave improved performance over TFTP derivatives. The response was twofold higher for all analytes evaluated. Retention times were slightly lower for the PFTP derivatives, confirming a trend of increased volatility with increased fluoro-substitution. TFTP reagent had the additional drawback of containing significant levels of methyl-TFTP and ethyl-TFTP, and an additional clean-

up step was mandatory to improve the blank level. Two strategies were employed: (a) purging the stock solution with an inert gas (nitrogen) in order to eliminate the volatile species or (b) fractionating on a C_8 SPE cartridge to selectively retain and remove them. Although it was not possible to achieve a quantitative elimination, both approaches were suitable to reduce these species to manageable levels for trace analysis. On the other hand, PFTP having a similar purity of 97% compared to TFTP did not require an additional clean-up step. Methyl-PFTP was detected in blank preparations, but at levels significantly lower than 1 $\mu g\,g^{-1}$ standard using the final experimental conditions. On the basis of these results, PFTP was selected as the most suitable reagent for the determination of AAs for HS–GC–MS. PFTP derivatives exhibited suitable properties in terms of selectivity and volatility. These properties have not been explored deeply for analytical purposes and only one application has been reported recently that employs PFTP as reagent for the detection of diphenylarsenic acid in water samples [33]. The derivatization reaction applicable to mesylates, besylates, tosylates and sulfates is given in Table 1. Note that for the same R_1 group in the different series, the same derivative is formed. It is therefore not possible with the described method to differentiate between AAs across the range of acids. Knowledge of the synthetic route, however, allows a targeted approach to the determination, and control of AAs at points in the synthesis relevant to the potential formation.

For a generic AA determination method, a solvent is required that readily dissolves pharmaceutical compounds in the range of 5–50 $mg\,mL^{-1}$. Moreover, the selected solvent should provide a high derivatization yield and maximise the partitioning of the derivatives into the HS. The solubility and yield were considered related, favouring a polar aprotic solvent. The yield and the HS partitioning were also clearly related, together contributing to the observed response, but in this case the relationship was antagonistic. As stated to maximise the yield, a polar aprotic solvent is required [34] but, on the other hand, to push the non-polar derivatives into the headspace, a proportion of water is required. Protic solvents, however, are generally not suitable for S_N^2 reactions, and therefore water should have a negative impact on the yield. Nonetheless, water proved to be a good solvent indicating that the partitioning of the derivatives was the dominant factor over the yield in the response observed. Water only would however not satisfy the solubility criteria of an ideal solvent. The lypophilic solvents tested in a 50/50 ratio with water, DMSO showed the best performance because of the highest dielectric constant value ($\varepsilon = 48$), favouring S_N^2 reactions (Fig. 1a). A more detailed study for water/DMSO mixtures showed higher enrichment factors when smaller amounts of DMSO were present (Fig. 1b).

In general, methyl-, ethyl- and isopropyl-AA recoveries were strongly improved when 12–25% of DMSO was used as a solvent mixture while the sulfates were less influenced. Di-isopropyl sulfate showed the highest yield with a DMSO/water ratio of 1. Limits of detection and quantification were calculated for a solvent mixture of 50% DMSO–water, a blank pharmaceutical sample of 50 mg and a spiking concentration of 1 ppm. The data are summarized in Table 2.

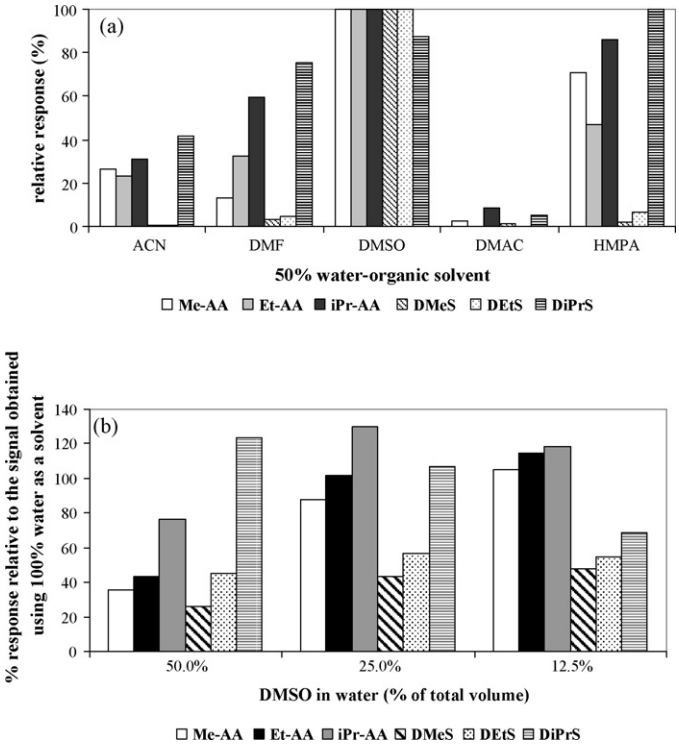


Fig. 1. Effect of solvent composition on response for *in situ* derivatization–HS–GC–MS using pentafluorothiophenol as derivatization agent with different alkylating agents at (a) 50% water–organic solvent (acetonitrile, ACN); dimethylformamide, DMF; dimethyl sulfoxide, DMSO; *N*,*N*-dimethyl acetamide, DMAC; hexamethyl phosporamide, HMPA) and (b) different DMSO–water ratios.

Although these conditions were not giving the highest response, they are suitable to be applied as a generic analytical method for pharmaceutical products, as polar and non-polar matrixes are covered. Eventually, the analytical approach can be tuned to select the most appropriate DMSO/water ratio to maximise the response of the AA of interest. This solvent combination exhibits a practical range of polarity to dissolve a broad spectrum of pharmaceutical products.

Reaction temperature (22–105 °C) and time (0–60 min) were optimised using a factorial design. Response surfaces were determined for each AA. The reaction rate trend was Me-AAs > Et-AAs ≫ *i*Pr-AAs. Optimum conditions for each AA

Table 2
Analytical quality parameters obtained for the proposed method

Analyte	LOD[b] ($ng\,mg^{-1}$)	LOQ[b] ($ng\,mg^{-1}$)	R.S.D.[c] (%)
Me-AAs[a]	0.11	0.31	5.0
Et-AAs[a]	0.03	0.06	7.1
*i*Pr-AAs[a]	0.07	0.15	4.2
DMeS	0.08	0.27	6.5
DEtS	0.04	0.09	2.8
D*i*PrS	0.12	0.30	10.0

[a] Alkyl (methyl, ethyl and isopropyl) alkylating agents (besylates, tosylates and mesylates).
[b] Sample amount 50 mg, DMSO/water ratio 1:1, concentration relative to sample amount.
[c] $n = 5$ replicates at 1.0 ppm spiked level relative to sample amount.

were different. Compromise conditions which kept analysis time to a minimum, but allowed detection of each AA at a low-level were finalised upon. The temperature selected for the derivatization stage was also the optimum for HS analysis (105 °C), and the equilibration time was set at 15 min in order to obtain high reaction yields for Et-AAs and *i*Pr-AAs.

It is well known that alkyl fluorides, esters, alcohols, ethers and amines do not undergo S_N^2 reactions under normal circumstances [34]. Since residual alcohols can occasionally be present in pharmaceutical matrixes in which an AA may have formed, it was necessary to confirm this from a trace analysis perspective. The specificity of the derivatization was assessed in the presence of methanol, ethanol and isopropanol up to a concentration of 5% relative to a typical test sample. No interferences were observed from any of the alcohols. Although this demonstrated the specificity of the determination from a group of the most likely impurities to be present in a matrix, it is recognised that specificity should be judged for each matrix independently. A positive result should therefore be appropriately confirmed.

3.2. Headspace–gas chromatography–mass spectrometry

Two injection volumes (1 and 3 mL) were assessed for HS sampling. Three milliliter was preferred for sensitivity reasons. Initially, the increased quantities of solvent injected for 3 mL compared to 1 mL caused band broadening of the analytes and proper selection of the column in combination with adjusting the split ratio was necessary. Different HS equilibration times were tested, and 15 min was demonstrated to be optimum. Increasing the HS temperature increased the response, up to 105 °C. A vial pressure of 16 psi was required to avoid back flush into the HS gas lines post-equilibration. The split flow contributions from the GC and the headspace sampler were high enough to facilitate rapid and efficient transfer of the sample to the column and to avoid band broadening, keeping sensitivity loss to a minimum.

Different columns were evaluated: a CB 624 (25 m × 0.150 mm i.d. × 0.84 μm, from Varian, Oxford, UK); ZB-5 ms (20 m × 0.18 mm i.d. × 0.18 μm from Phenomenex, Torrance, CA, USA); HP-5 (30 m × 0.32 mm i.d. × 0.25 μm from Agilent) and a RH-624 (30 m × 0.32 mm ID × 1.8 μm from Capital Analytical Ltd., Leeds, UK). The latter column, a 6% cyanopropylphenyl, 94% dimethylsiloxane with a 1.8 μm film thickness exhibited the best performance. Sufficient resolution of the analytes and the solvent was achieved when a 3 mL loop volume was employed. Previous work showed that this column was the most suitable for perfluorinated compounds [32]. This column exhibits a high retention for volatile compounds, but retention times were short by using a fast oven temperature ramp rate. The initial oven temperature was 60 °C to focus analytes at the head of the column. This initial oven temperature also kept cycle times to a minimum.

Negative chemical ionisation (NCI) MS and electron capture detection both provide very high responses for perfluorinated compounds ([32] and data not shown) however electron impact ionisation (EI) in the SIM mode was selected because it has advantages in terms of robustness and easier transferability to other laboratories. Moreover, EI provided strong molecular ions

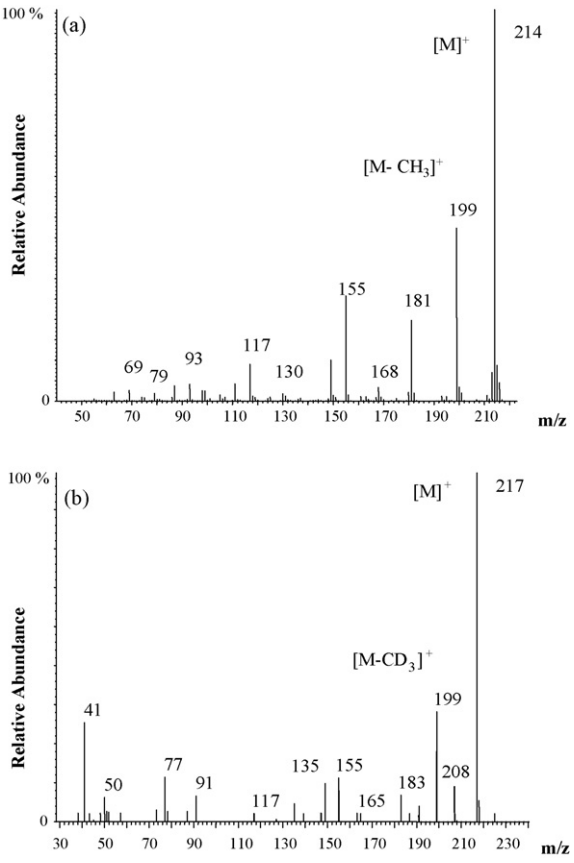


Fig. 2. EI mass spectra of (a) methyl-AAs and their corresponding (b) deuterated IS after *in situ* derivatization–HS–GC–MS using pentafluorothiophenol as derivatization agent.

for all solutes and deuterated analogues. This is illustrated in Fig. 2 for the pentafluorothiophenol derivative of methyl-AAs and the corresponding deuterated analogue.

Deuterated analogues are the most suitable compounds for high accuracy and precision, since they can compensate for the variables in the derivatization, and extraction/injection processes. Another advantage is that deuterated internal standards can provide assurance against the possibility of reporting false negative results. Only single ions were monitored for the internal standard and the analytes (see Table 1). The use of qualifying ions was precluded by the sampling rates achievable on the MSD.

3.3. Sample analysis

The analytical parameters of LOD, LOQ and repeatability have been evaluated, the results are summarised in Table 2. The linearity of the response ratio (analyte: IS) was assessed, without a matrix, over an analyte range of 0.25–50 μg g^{-1} relative to a test sample. Correlation coefficients of $r > 0.997$ were achieved for all AAs tested. Repeatability was studied by five replicate experiments at the 1.0 μg g^{-1} (1 ppm) level. Relative standard deviations (R.S.D.) in the range of 2.8–10.0% were achieved. Fig. 3 shows a typical chromatogram at the 1 ppm level of the derivatized analytes evaluated, showing that it is possible to separate and determine the alkyl derivatives of PFTP in

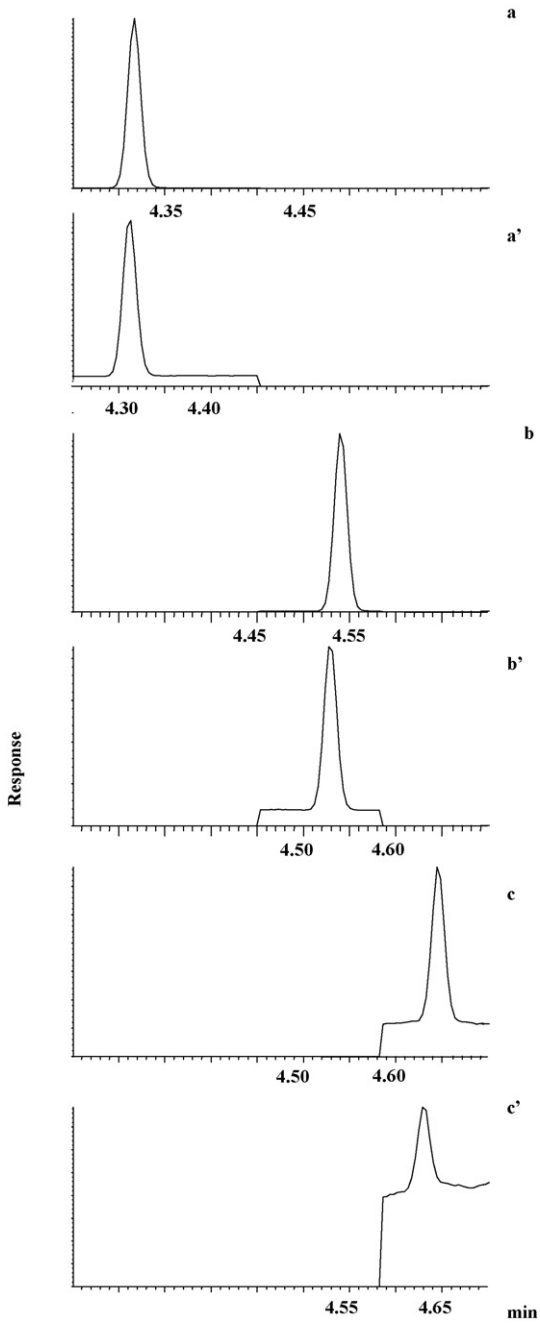


Fig. 3. HS–GC–MS selected ion chromatograms showing the PFTP derivatives: (a) methyl ($m/z^+ = 214$); (b) ethyl ($m/z^+ = 228$); (c) isopropyl ($m/z^+ = 242$), and their corresponding deuterated IS's (a′) ($m/z^+ = 217$), (b′) ($m/z^+ = 233$) and (c′) ($m/z^+ = 249$). Selected analyte was alkyl tosylate at 1.0 μg g^{-1} (1 ppm) relative to 50 mg of sample.

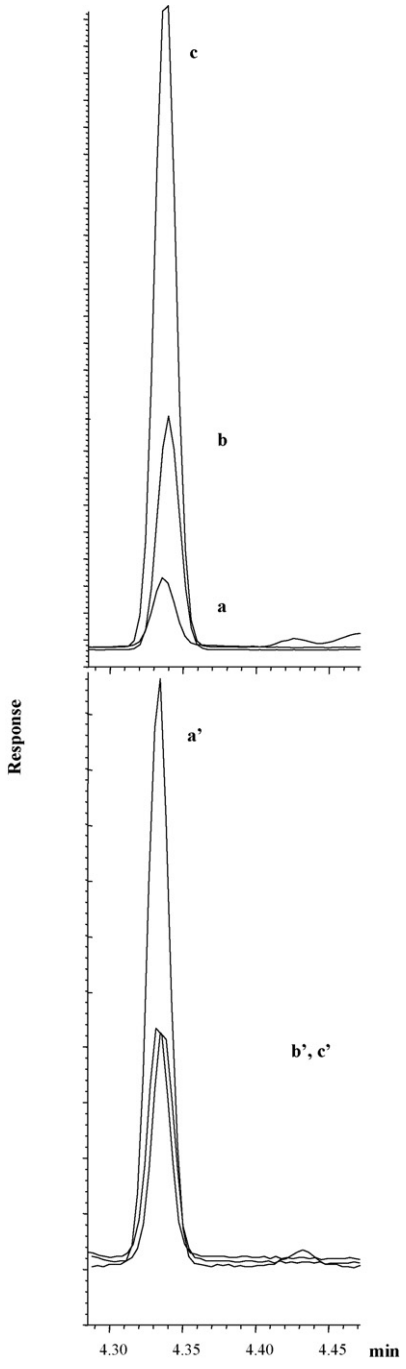


Fig. 4. HS–GC–MS selected ion chromatograms at $m/z^+ = 214$ for methyl tosylate derivative and $m/z^+ = 217$ for the deuterated internal standard. Solvent mixture: dimethyl sulfoxide-water 1:1. (a) Background level for the PFTP methyl derivative, (b) analysis of a pharmaceutical sample (40 mg) containing 1 ppm methyl tosylate and (c) analysis of the pharmaceutical sample (b) spiked at the 2.4 μg g^{-1} level. Illustration of the loss of response for the same concentration of deuterated internal standard added due to matrix effects (a′), blank injection (b′ and c′) and injection of samples (b and c).

less than 5.0 min. LOD, LOQ and precision values were matrix dependent.

Different pharmaceutical matrixes were successfully analysed applying the analytical methodology developed. Samples under investigations had been in contact with different alcohols (methanol, ethanol and/or isopropanol) and acids (e.g. *p*-toluenesulfonic acid and benzenesulfonic acid), during the manufacturing process. In general, AA concentrations were below the LOD values (e.g. sample B), except in one instance (sample A), where methyl tosylate was detected (see further in Fig. 4). Different quantification procedures such as external calibration (EC), internal calibration (IC) and standard addition (SA) with or without internal standard were assessed. SA with internal standards was found to be the most suitable method of quantification since recoveries were generally matrix dependent.

SA is a common procedure in the pharmaceutical industry for determination of analytes at trace levels because it compensates for the matrix effects typically encountered. For example, sample B gave acceptable recoveries relative to external standards (85–100%) for all the analytes evaluated, but sample A gave low recovery (40%) of methyl tosylate (Fig. 4), which had a detrimental effect on the LOD and LOQ for this matrix (0.19 and 0.41 $\mu g\,g^{-1}$, respectively). For ethyl and isopropyl tosylate in sample A, a matrix effect was not significant (recoveries > 93%). In general, the matrix effects encountered decreased recoveries, but in some instances the matrixes enhanced recovery. In the latter case the matrix seems to be contributing to a salting out effect of the derivatives, improving their partitioning into the HS. Despite these effects LOQs in all samples analysed with a sample loading of ~50 mg were less than 1.0 $\mu g\,g^{-1}$ (1 ppm) for each AA.

4. Conclusion

A simple, fast, reliable and versatile generic analytical method has been developed for the determination of a broad spectrum of trace level AAs in a wide variety of pharmaceutical matrixes. The method gives information on the alkyl groups of the AAs and does not differentiate between AAs. In this generic method, each analytical step is closely interrelated, but special care has been taken to maintain optimum conditions (selectivity, analysis time and efficiency) for a trace level determination. Solvent selection was a critical parameter in development, and was a key step in making the final method applicable to a broad range of pharmaceutical developmental compounds. A range of solvents, known for their excellent solvating power in this field, were evaluated but DMSO and water were found to be optimum. The ratio of water/DMSO was evaluated to optimise the derivatization yield and consequently the sensitivity of the method. The derivatization step, using PFTP, facilitates and increases the selectivity of the determination in terms of the limited number of side reactions possible, as well as allowing analyte-matrix separation by HS extraction. While adding additional complexity and cost, the use of deuterated internal standards was required to achieve adequate performance of the analytical method.

Impurities that are known or suspected to be toxic present a special concern during drug development. The goal for investigation of such impurities is to demonstrate that they are undetectable or below an appropriate level of concern. A targeted method is usually required for investigation of these impurities. The method has been shown to be suitable for determination of these impurities and can be applied as a routine method efficiently, ensuring product quality and safety, and meeting current regulatory expectations.

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