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Pharmaceutical applications of high-performance liquid chromatography interfaced with Fourier transform infrared spectroscopy

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ABSTRACT

Application of the solid-phase extraction interface for high-performance liquid chromatography coupled with Fourier transform infrared spectroscopy to problems of pharmaceutical interest is described. A diagram of the interface and description of its use is given. Application of the system to the identification of isomers and degradation products in active ingredients, and contaminants in product excipients are described. The advantages of the use of the solid-phase extractor interface, and its use in on-column and on-extractor preconcentration to increase sensitivity are described.

INTRODUCTION

Infrared (IR) spectroscopy is a powerful technique for the identification of organic compounds. The ability to obtain structural information on compounds, along with its simplicity make it the technique for choice for many qualitative determinations. Furthermore, Fourier transform infrared (FT-IR) spectroscopy is sensitive enough to allow the technique to be used for trace analysis.

The qualitative attributes of FT-IR compliment those of chromatography, which is essentially a quantitative method, and substantial effort has been devoted to interfacing these techniques. While success has been achieved in coupling gas chromatography with FT-IR, less success has been achieved with high-performance liquid chromatography (HPLC). The major problem with HPLC–FT-IR is the interference caused by the liquid mobile phase. This is particularly true for reversed-phase chromatography, which accounts for the large majority of HPLC separations, because water and commonly used polar organic solvents present in the mobile phase strongly absorb in the IR spectral region.

The interface is the critical component in the HPLC–FT-IR system, and the success or failure of the technique is determined almost exclusively by its performance. HPLC–FT-IR interfaces can be classified into two general categories; continuous and analyte capture type. Continuous techniques employ some type of flow cell in order to introduce the analyte into the spectrometer. Three types have been employed: the conventional flow cell using a polymer or lead spacer to set the cell path length [1], the cylindrical cell [2], and the attenuated total reflectance (ATR) flow cell [3]. For direct measurement of reversed-phase HPLC eluents the attenuated total reflectance cell has been used with some success [3–5].

Continuous methods using aqueous mobile phase elimination have been utilized for HPLC–FT-IR. Taylor and co-workers [6–8] have developed a liquid–liquid extraction interface for use with reversed-phase HPLC. In this approach the analytes were extracted from the mobile phase by mixing

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post-column with a stream of IR transparent, water immiscible solvent. The immiscible solvent, typically CCl_4, C^2HCl_3 or $CHCl_3$, was separated from the segmented eluent stream by a membrane separator, and passed through a flow cell for detection.

Although continuous HPLC–FT-IR systems are advantageous in that they can provide real time chromatographic information, the real usefulness of the HPLC–FT-IR lies in its ability to provide spectral information on analytes. To this end techniques employing analyte capture have been very successful. In general, two basic analyte capture approaches have been employed. In the deposition technique the eluent is deposited on a continuously moving, IR transparent, inert substrate from which the eluent can be easily removed by evaporation. The substrate containing the individually captured analytes is transferred to the spectrometer for spectral analysis. In the solid-phase extraction technique the analytes are collected on a solid adsorbent from which the mobile phase can be eliminated by evaporation. The analytes are eluted from the adsorbent material with an IR transparent solvent and transferred to the spectrometer for spectral analysis. Of these two techniques the deposition method has been the most popular, and some significant advances have been made recently.

The deposition techniques are based on the work of Kuehl and Griffiths [9] and Jinno and co-workers [10–14]. In these original works systems for normal-phase HPLC were described.

Kalasinsky and co-workers [15,16] used a post-column system which converted water to acetone and methanol by reaction with 2,2-dimethyloxypropane. The analytes were then collected by deposition of a nebulized, reacted stream of eluent onto a KCl substrate. This system was used with both microcolumns and packed columns, with detection limits around 1 μg when diffused reflectance analysis was employed.

Analyte capture by deposition on a reflective surface is a technique which shows great potential for development into a sensitive analytical method [17–21]. These techniques which typically employ evaporation of the mobile phase using a heated evacuated chamber, allow spectra to be obtained at very low levels. Identification quality spectra at the 0.5 ng level have been reported [21].

An alternate to the deposition methods for analyte capture based on post-column solid-phase extraction has been described [22,23]. In this technique the analyte of interest was extracted on a small column after post-column dilution of the mobile phase with water. The extractor column was dried with nitrogen and the analyte eluted into a conventional flow cell with an IR transparent solvent. Because of the concentration effect of the post-column extraction, sub-microgram quantities of analyte could be detected, with microgram quantities used to obtain identifiable spectra.

The purpose of this paper is to describe the application of the solid-phase extractor HPLC–FT-IR interface for pharmaceutical applications. Application of the system to the identification of isomers and degradation products in active ingredients, and contaminants in product excipients are described. The advantages of the use of the solid-phase extractor interface, and its use in on-column and on-extractor preconcentration to increase sensitivity are described.

EXPERIMENTAL

The chromatographic system used was a Waters (Milford, MA, USA) Model 204 HPLC with two Model M6000 pumps, a Model 680 gradient controller, a Model 440 absorbance detector, and a Model 712 WISP autoinjector. Chromatographic data collection was performed on a Hewlett-Packard (Valley Forge, PA, USA) HP-3350A LAS computer.

The IR spectrometer used was a Nicolet Instruments (Madison, WI, USA) Model 20DXC with a deuterated triglycine sulfate (DTGS) detector. Spectral acquisition was performed on a Model 620 workstation. Data processing was accomplished using Nicolet DXFTIR software. No beam condenser or spectral enhancement devices were used in this work.

The HPLC–FT-IR interface consisted of a Waters M6000 pump for pumping the IR transparent solvent through the extractor, an LKB 2150 HPLC pump (Paramus, NJ, USA) for pumping the mobile phase diluent (water for reversed-phase separations), a Valco Instruments (Houston, TX, USA) 6-port, 2-position valve, a Valco Instruments 12-port manifold valve, a Valco Instruments 10-port, 2-position valve, and a Valco Instruments serial

valve interface. A Specac micro flow cell (Aries, Concord, MA, USA) with lead spacers and CaF_2 windows was used to introduce the sample into the spectrometer. Extractor columns, 20 × 2 mm I.D. (Upchurch Scientific, Oak Harbor, WA, USA), were packed with C_{18} packing (PrepSep C18, Fisher Scientific, Pittsburgh, PA, USA).

All solvents used were HPLC grade and filtered through type HVLP membrane filters (0.45 μm) (Millipore, Bedford, MA, USA) prior to use.

RESULTS AND DISCUSSION

Although many different interface configurations have been developed for HPLC–FT-IR, no one system has been shown to be generally applicable for reversed-phase chromatography. This is demonstrated by the fact that while there has been a significant amount of work done in this area, there are few publications on applications of the technique to actual analytical problems. No one design can be recommended for general use. The selection of a particular interface configuration will depend on the particular application.

Table I lists those parameters which should be considered in selecting an HPLC–FT-IR interface. Of the practical considerations, the amount of automation required of the system to handle the analytes and the expertise of the analyst are most important in deciding on the type of interface required for a particular application. As shown above some of the interface designs are complicated to use. Therefore, these designs require a high skill level for an analyst. Also, the complicated nature of these interfaces makes them more expensive and time consuming for analysis.

From a chemical perspective, it is the analyte stability and sensitivity requirements which determine the choice of interface design. In cases where unstable analytes are being determined, the selection of the interface design can be critical. Most of the deposition interfaces use elevated temperature to aid in mobile phase evaporation. Also transfer of the substrate from the deposition apparatus to the spectrometer can expose the analyte of oxygen, light, and moisture under uncontrolled conditions. These may not be acceptable in some situations. This is the case in our laboratory where we routinely work with labile compounds.

TABLE I

PARAMETERS FOR INTERFACE SELECTION

Practical	Time
	Cost
	Analyst expertise
	Manual *vs.* automated
	Instrumentation availability
Chemical	Analyte stability
	Sensitivity required

Sensitivity requirements of the assay also determine the interface design selection. One of the major design objectives of HPLC–FT-IR instrumentation has been to lower the detection limit as far as possible. While high sensitivity may be required for some applications, it has placed severe limitations on the use of the technique. There are many situations where the analyst is not concerned with detecting a low absolute amount of analyte, such as ng of pg quantities, but with detecting a low relative amount, such as % or μg/g levels. This is true in the pharmaceutical industry, where it is required to identify impurities and degradation products in drug substances and finished products. These analytes are present in the sample at levels as low as 0.05% (w/w). At these levels the analyte may be below the limit of identification of all but the most sensitive HPLC–FT-IR interface. However, design of the separation may make it possible to have μg or even mg quantities of analyte introduced into the spectrometer after separation. This gives the analyst a choice of utilizing one of the simpler, but less sensitive interfaces. This has been the case in our laboratory.

Based on the above considerations the post-column solid-phase extraction interface was developed and used in our laboratory to investigate a variety of problems. This system was chosen for the following reasons. First, the system is automated so that it can be simply operated by selection of menu items from an in-house written program. Second, it allows the analyte to be automatically transferred from the chromatograph to the spectrometer. This minimizes analyte exposure to oxygen, light or heat. Third, the system is capable of the required sensitivity to obtain spectra of impurities and degradation products. Fourth, the system does not require dedicated instrumentation.

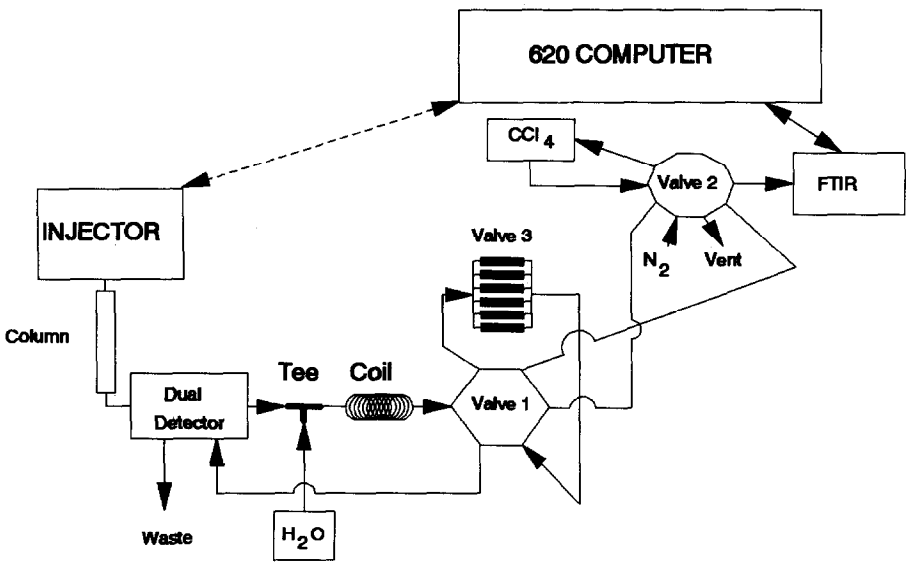

Fig. 1. Diagram of the automated solid-phase extraction interface for HPLC–FT-IR.

Fig. 1 shows a schematic diagram of the HPLC–FT-IR system used in our laboratory. It is designed so that the chromatograph and spectrometer can be operated independently. This has the advantage of allowing chromatographic analyses or other operations (*i.e.*, solvent change over, separation development) to be performed on the chromatographic side while spectral data acquisition and manipulation is being performed on the spectrometer. The system could be controlled entirely from the spectrometer side, if desired.

The extractor interface consists of three valves and a solenoid switch, each of which can be controlled independently by either the chromatograph or the spectrometer. Two pumps are incorporated into the interface. Water is introduced with one

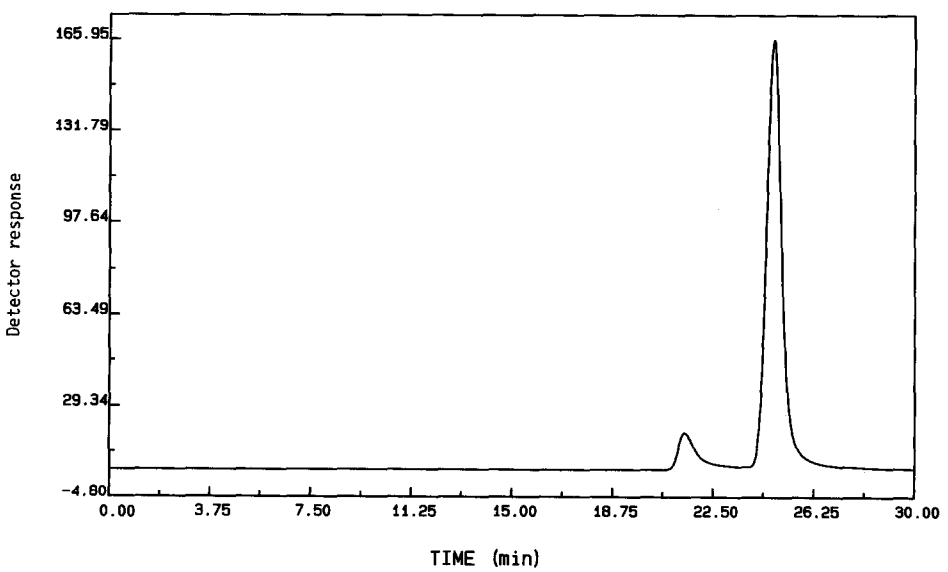

Fig. 2. Chromatogram of homosalate. Mobile phase: acetonitrile–water (70:30, v/v). *y*-Axis in arbitrary units.

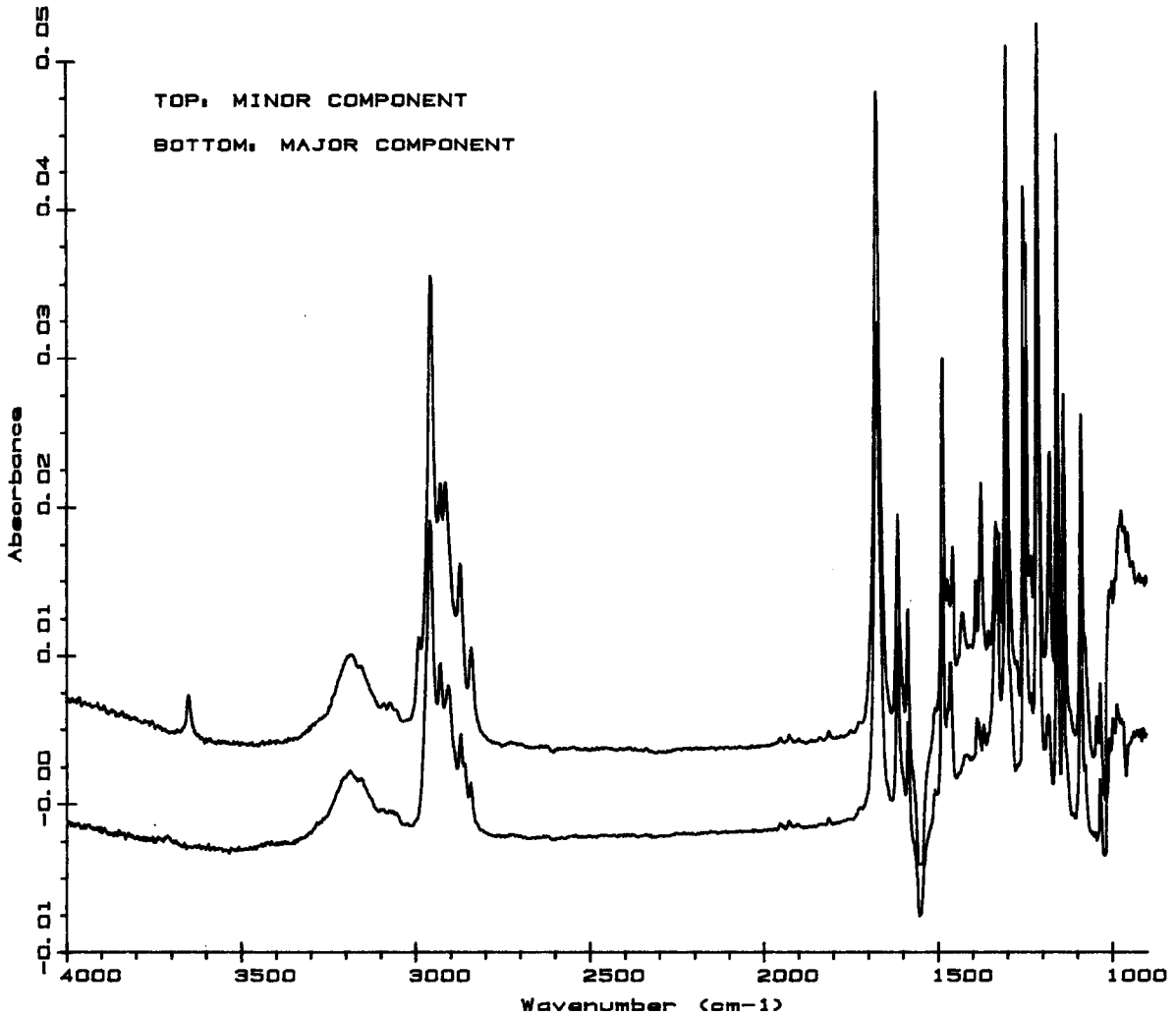

Fig. 3. Overlay spectra of homosalate components.

pump into a mixing tee connected to the outlet of the column. This is used to dilute the reversed-phase eluent to allow extraction of the analyte on the reversed-phase packing of the extractor. A short mixing coil of about 10 cm of 0.8 mm I.D. stainless steel tubing is used to increase mixing of the mobile phase with the water diluent. The second pump is used to continuously pump the IR transparent solvent used to elute the extracted analytes from the extractor. In order to conserve solvent it is normally recycled back to the solvent reservoir.

For normal operation the system is used as follows. Several initial chromatograms are obtained in order to optimize the separation and determined the retention times of the analytes of interest. The extractor manifold is then conditioned by passing solvent through each extractor for some pre-determined time. This is typically done for 3 min at a flow-rate of 1 ml/min of eluent plus 2 ml/min of water from the diluent pump. The conditioning cycle is controlled by the spectrometer. Analyte extraction is performed by injection of the sample on the column and switching to the extractor manifold prior to the pre-determined retention time of the

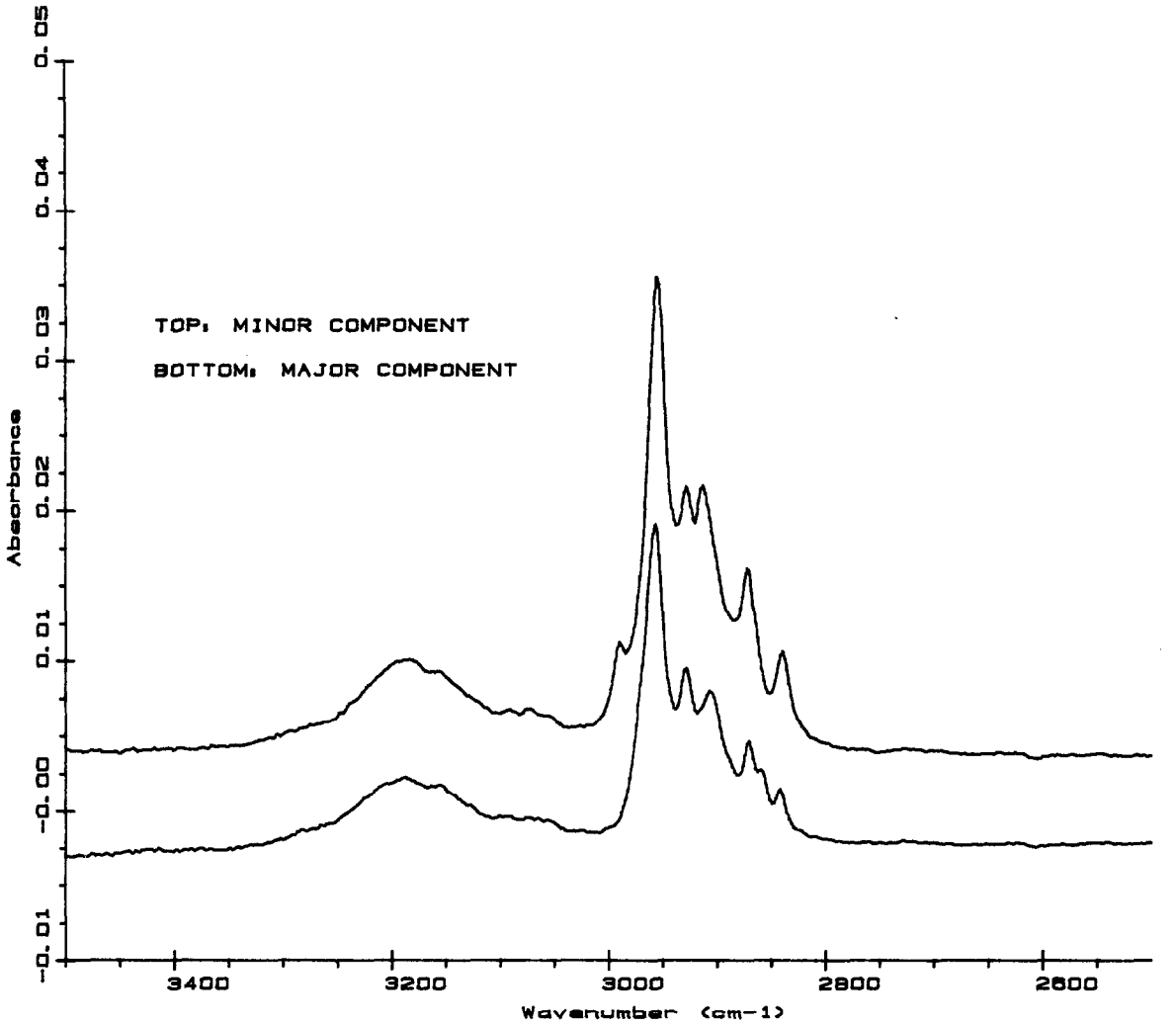

Fig. 4. C–H stretching region of spectra of homosalate components.

peaks of interest. This operation is controlled by the chromatograph. Although an absorbance detector is used on the chromatograph, is is not used to trigger valve switching. It is used initially to determine the retention times of the analytes of interest. It is also used during the extraction operation to determine the extraction efficiency by monitoring the flowing stream into and out of the manifold.

After extraction of the analytes control of the interface is done by the spectrometer. First, the manifold is dried by passing nitrogen at $6.1 \cdot 10^5$ Pa through each extractor. A short pressure equilibration cycle is built into the program in order to release trapped nitrogen from the manifold. At this point the analytes are ready for spectral analysis. This is accomplished by individually back-flushing each extractor with an IR transparent solvent in order to elute the analyte and introduce it into the flow cell. At this point spectra are obtained and saved for later analysis. Typically, thirty coadded scans at 4 cm^{-1} resolution are obtained for each sample.

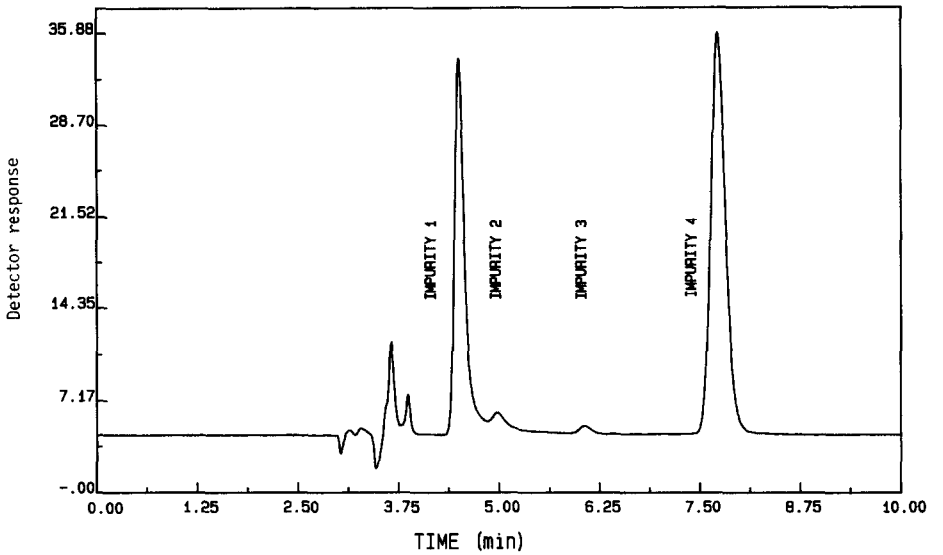

Fig. 5. Chromatogram of impurities in propylene glycol. Chromatographic conditions: acetonitrile–water (25:75, v/v), at time = 0 go to acetonitrile–water (90:10, v/v) in 5 min, hold 12 min. *y*-Axis in arbitrary units.

Applications

3,3,5-Trimethylcyclohexyl 2-hydroxybenzoate (Homosalate) is an ester of salicylic acid. This compound is important in the dermatological and cosmetic product areas because it is the reference compound required for the United States FDA Sunscreen Monograph [24] method for the determination of Sunscreen Protection Factors (SPF) for topically applied sunscreen products. Fig. 2 shows a chromatogram of this material. Upon reversed-phase separation of homosalate two peaks are observed. Although these peaks have similar UV spectra, the absorbance ratios differ slightly. Also, lot to lot and sample to sample variations in the ratio of the two components have been observed. The amount of the lesser component can vary by as much as 100%.

The solid-phase extractor interface was used to obtain IR spectra of the two components in order to identify them. Fig. 3 shows an overlay of the spectra obtained for the two components. These closely matching spectra indicate that both are isomers of homosalate. This was later confirmed by molecular mass determination of each component by LC–mass spectrometry (MS).

Fig. 4 shows an expanded view of these overlaid spectra in the C–H stretching region where the main differences between the two are observed. Inspection of the spectra in this region shows that there are three sets of doublets. For both components two of these doublets are observed at nearly the same frequencies and correlate well with known C–H stretching modes [25]. The methylene C–H stretch is observed at 2928 and 2840 cm^{-1}. The methyl C–H stretch is observed at 2957 and 2872 $^{-1}$.

A third set of C–H stretching bands is observed in the two spectra, but they do not appear at the same frequency in the two spectra. In the major component's spectrum these are observed at 2906 and 2859 cm^{-1}. In the minor component's spectrum these bands are shifted to 2990 and 2913 cm^{-1}.

Homosalate is a substituted cyclohexane with two methyl groups in the 3 position and one in the 5 position of the ring. These methyl groups and the methylenes of the cyclohexane ring are the only aliphatic C–H groups on the molecule. Therefore, the methylene C–H stretching must arise from the cyclohexane CH_2. The other two doublets must arise from the methyl substituents on the ring.

The differences in these spectra suggest that these components are conformational isomers of cyclo-

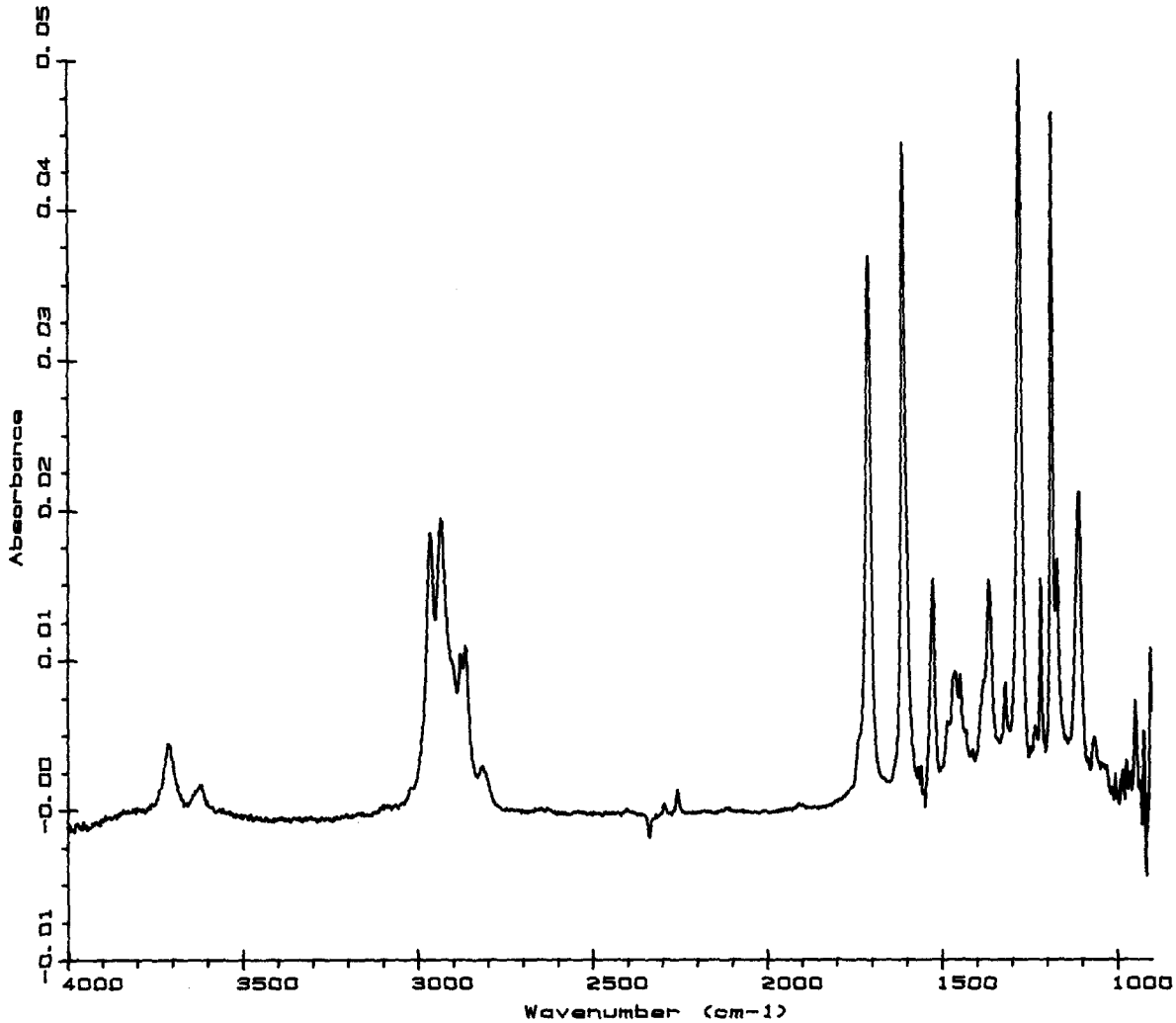

Fig. 6. Spectrum of impurity 4 in propylene glycol.

hexane. The spectra clearly indicate that these components differ in the cyclohexane portion of the molecule. The shift of the methyl C–H stretch could be the result of steric effects between an axial methyl group and the bulky salicylate group occupying the axial position on the cyclohexane ring in one component compared to its presence in the equatorial position in the other component. However, exact assignment of the structure to the components cannot be made with the data available.

Another application in which HPLC–FT-IR is useful is for the identification of impurities in product excipients. Fig. 5 shows a chromatogram of impurities in a sample of propylene glycol. At least four impurities are seen in the chromatogram. These are not observed in chromatograms of clean propylene glycol. Based on the chromatographic response of the impurities it was concluded that the propylene glycol was contaminated at a very low level. No detectable spectra could be obtained for single injections of up to 100 μl of 1:1 dilution of propylene glycol in water. Therefore a preconcentration technique was employed to obtain the IR spectra of impurities.

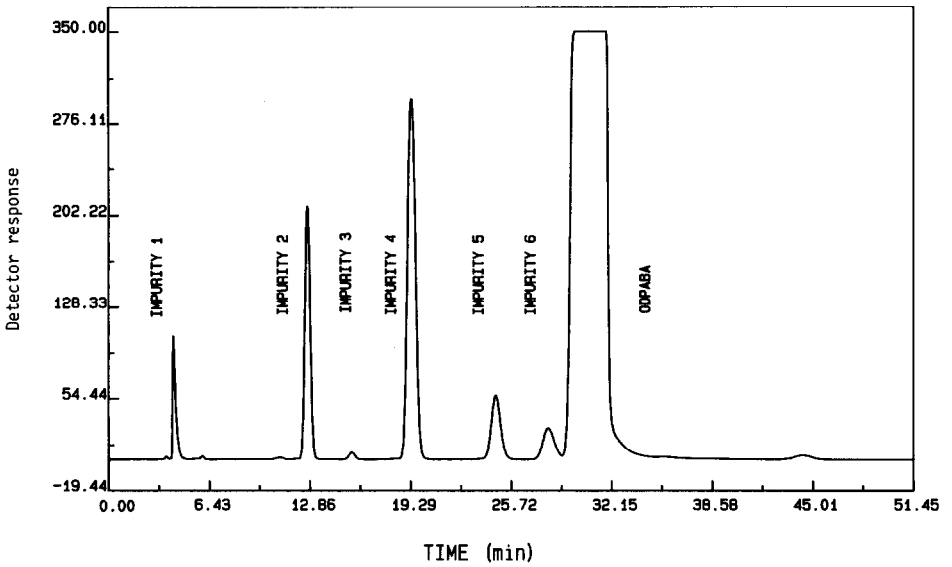

Fig. 7. Chromatogram of impurities in octyl dimethyl *p*-aminobenzoate. Mobile phase: tetrahydrofuran–water (55:45, v/v). *y*-Axis in arbitrary units.

The following procedure was used for this analysis. The procedure employed on-column concentration of the impurities by making nine 200-μl injections of 1:1 propylene glycol in water. The impurities were eluted by gradient elution, and three of the four impurities captured on the extractor. This process was repeated four times, so that a total of 36 injections were collected on the extractor interface. The extractor was dried under nitrogen for 7 min, and the impurities eluted into the spectrometer using CCl_4.

Fig. 6 shows the spectra obtained for impurity 4. Clearly a spectrum which is suitable for identification is obtained using the preconcentration technique outlined above. Based on this spectrum impurity 4 was determined to be octyl dimethyl *p*-aminobenzoate (ODPABA). This was confirmed by matching retention times of the impurity with that of a sample of ODPABA, and by molecular mass determination by LC–MS.

Analysis of the contaminated propylene glycol found ODPABA to be present at a concentration of 19.4 μg/g. Identification of this impurity at this low level demonstrates how the extraction interface can be used to identify low levels of impurities in product excipients. This is an important area of application in the pharmaceutical industry.

ODPABA is a chemical of some significance in dermatology. It is used as an active ingredient in topical sunscreen formulations. *p*-Aminobenzoate (PABA) and its derivatives can undergo degradation to produce impurities [26,27] which may be biologically active [28,29]. Although it has been replaced in many products by other sunscreen actives, it is still used in a variety of topical sunscreen formulations.

Fig. 7 shows a reversed-phase chromatogram of ODPABA. Several components, the largest of which is at about 1%, can be seen in this chromatogram. The HPLC–FT-IR extraction interface was used in the identification of degradation products in ODPABA.

Fig. 8 shows the IR spectra obtained for two of the components in ODPABA. Both spectra contain absorption bands characteristic of amines. The spectra for impurity 4 show bands at 3440, 2820 and 1272 cm^{-1}. These are indicative of an aromatic molecule containing a secondary amine [25]. The spectra for impurity 2 show bands at 3500, 3408, 1622 and 1272 cm^{-1}. These are indicative of an aromatic molecule containing a primary amine [25]. Furthermore, the rest of the spectra of these two components match that of ODPABA, indicating that the basic structure of the molecules is the same.

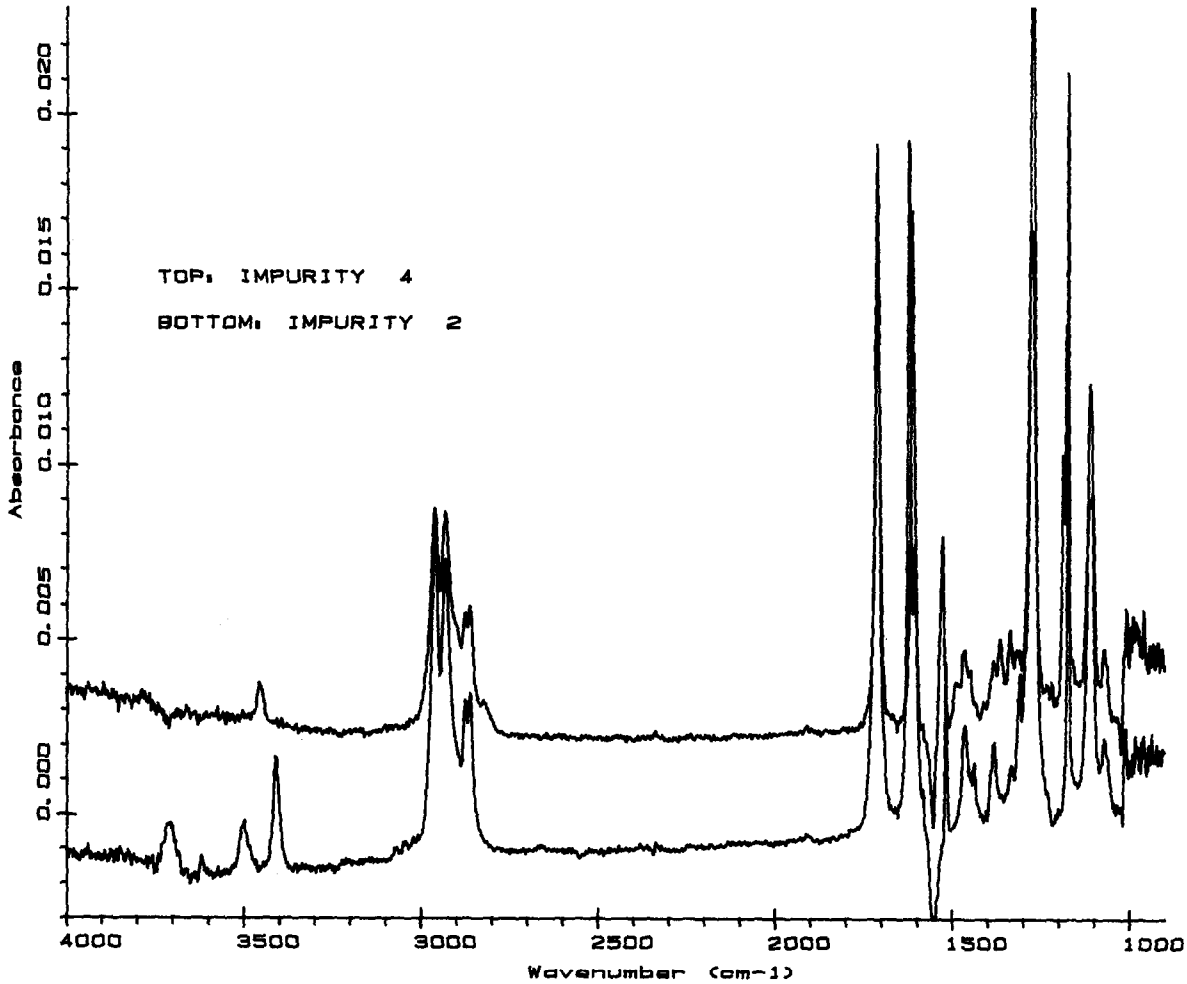

Fig. 8. Overlay spectra of impurities in octyl dimethyl *p*-aminobenzoate.

Based on these spectra impurity 2 was identified as the primary amine degradation product of ODPA-BA, octyl PABA, and impurity 4 was identified as the secondary amine degradation product of OD-PABA, octyl N-methylPABA. These observations were later confirmed by LC–MS.

CONCLUSION

HPLC–FT-IR with a solid-phase extraction interface has been used successfully to investigate a number of problems of pharmaceutical interest. The system has been used to identify isomers, trace contaminants, and degradation products in active ingredients and product excipients. Its ability to provide high quality spectra make it useful for identification and structure elucidation of components in mixtures. Although the solid-phase extractor may not be the ideal interface for every situation, its ease of use and its flexibility make it well suited for the problems described above.

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