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# Analysis of impurities by high-performance thin-layer chromatography with Fourier transform infrared spectroscopy and UV absorbance detection in situ measurement: chlordiazepoxide in bulk powder and in tablets

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**Abstract**

On-line coupling of HPTLC and infrared spectroscopy enables the separation, detection and identification of impurities from the main substance. The decomposition products of the benzodiazepine chlordiazepoxide have been investigated both in raw materials and in pulverized tablets, that had been subjected to controlled thermal and hydrolytic stress treatments. The measurements of the diffuse infrared reflectance were carried out using a DRIFT unit. After thin-layer chromatographic development the impurities were detected with the help of the Gram–Schmidt procedure. The substance peaks were identified by calculating quasi-absorbance spectra and comparison with the reference spectra of the library. The reference substances were only required initially for compilation of the library. The method was used to determine the hydrolytic degradation product nordazepam, which is not specifically tested for according to the monographs of the Pharmacopoeias in addition to aminochlorobenzophenone and demoxepam. Quantitation was carried out densitometrically at 230 nm. The results obtained with the example chlordiazepoxide demonstrated the efficiency of the system, for it was possible to identify and quantitate the drug and its impurities down to 0.05%. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Detection, TLC; Pharmaceutical analysis; Infrared spectroscopy; Chlordiazepoxide

## 1. Introduction

The Pharmacopoeias frequently specify thin-layer chromatography (TLC) for tests concerning identity and purity [1]. The presence of impurities is an important criterion for evaluating the pharmaceutical quality and is necessarily done to determine the shelf-life after storage or stress tests [2]. Since chlordiazepoxide and its related compounds are clearly visible under short-wavelength UV light, the monographs generally prescribe the comparison of size and intensity of the sample spot and either the diluted sample solution (Ph. Eur.) or standard solutions (USP). This visual estimation is semiquantitative at the best and does not provide evidence of the identity. At present, the coupling of chromatographic methods with mass spectrometry prove to be most effective for the identification of unknown impurities, since structural and molecular mass information can be obtained from the tandem and full mass spectra [3]. However, the procedure often

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includes derivatization of the sample and is sometimes less suitable for routine analysis. Methods of quantitating the impurities of chlordiazepoxide employing high-performance liquid chromatography (HPLC), supercritical fluid chromatography (SFC) and gas chromatography (GC) are described in the literature [4–9]. A densitometric method for possible impurities has been developed on the basis of the USP monograph [10]. All these methods rely on retention time, $R_F$ value or the UV spectrum for the identification of unknown impurities. Although UV spectroscopy allows rapid quantitation, the paucity of bands in the spectrum means that structurally similar substances cannot generally be distinguished from each other [11]. The coupling of TLC with Fourier transform infrared spectroscopy (FT-IR), on the other hand, is distinguished by high selectivity. Its strength lies in the recognition and differentiation of closely related substances by comparison of the reference spectra previously recorded from the TLC plate. Hence, for the purpose of rapid identification and quantitation it is appropriate to combine these two spectroscopic techniques with modern TLC [12–17]. The subject of this publication is the application of diffuse reflectance IR spectroscopy in combination with high-performance (HP) TLC for the reliable identification of impurities, using chlordiazepoxide as an example.

## 2. Experimental

### *2.1. Reagents and standards*

TLC was performed on HPTLC plates precoated with a 200 μm layer of silica gel 60 $F_{254}$, with dimensions of 20×10 cm and glass support from Merck (Darmstadt, Germany). Methanol and ethyl acetate were of analytical reagent grade and purchased from Merck. The standards chlordiazepoxide (1), demoxepam (3) and nordazepam (4) were kindly supplied by Hoffmann-La Roche (Basel, Switzerland) or, in the case of 2-amino-5-chlorobenzophenone (2), obtained from Sigma (Deisenhofen, Germany). The chlordiazepoxide powder was a donation from Hoffmann-La Roche in 1989 and has been stored since then in a safe for controlled substances. The tablets [Limbatril tablets, Hoffmann-La Roche, Batch 00811, contents: 14.15 mg of amitriptylin (5) and 5 mg of chlordiazepoxide] were purchased from a local pharmacy.

### *2.2. Analytical procedures*

The standard stock solution was prepared by accurately weighing 5.0 mg of (1), (2), (3) and (4) into a 5-ml volumetric flask and diluting with methanol. Working solutions of 0.1 μg/μl were prepared by sequential dilution. The solutions were stored at 8°C and were stable under these conditions for several weeks.

A 100.0-mg amount of chlordiazepoxide was weighed into a 10-ml volumetric flask. After addition of 8 ml methanol, the solution was placed in a supersonic bath for 5 min and then made up to volume with methanol. A 50-μl volume of this solution was applied to the TLC plate. For the assay of chlordiazepoxide, the stock solution was diluted to a concentration of 0.1 μg/μl and 10 μl was applied.

For the assay of related compounds in Limbatril tablets, the tablets were crushed and 1.8 mg was transferred to a 5-ml volumetric flask, followed by the addition of 5 ml absolute methanol, mixing for 10 min in the supersonic bath and centrifugation for 3 min at 3000 rpm. A 50-μl volume of the supernatant solution was applied in 5-mm bands to the plate. For the assay of chlordiazepoxide, the stock solution was diluted to a concentration of 0.1 μg/μl and 10 μl was applied. The assay obtained from the tablet formulation was used as baseline value on the stability program.

During the stress test the powdered tablets were stored for a month in a desiccator above a saturated solution of sodium chloride (75% r.h.). The temperature was set at 50°C. The samples were taken after one day and thereafter once a week. With increasing amounts of degradation products, less volumes of the solution needed to be applied (10–30 μl) so that the substances could be quantified within the validated working range.

### *2.3. Chromatographic procedures*

Before use the plates were cleaned by development with methanol. Then the plates were dried in air and activated for 10 min at 160°C. Samples and

standards were applied to the plates by means of a Linomat IV (Camag) equipped with a 100-μl syringe; the band length was 5 mm, the (variable) application volume 10–50 μl, applied 10 mm from the bottom edge, 5 mm apart and developed with ethyl acetate as mobile phase to a distance of 60 mm, in a saturated linear development chamber. The development time was less than 17 min. The $R_F$ values were: 0 (5), 0.17 (1), 0.40 (2), 0.58 (3) and 0.81 (4). Only (2), (3) and (4) have been analysed.

Two-dimensional TLC was employed in order to achieve a higher concentration in the sample spots and therefore lower the limit of detection. The second development step was carried out with methanol to a height of the ends of the 5 mm bands.

### *2.4. Infrared measurement*

On-line HPTLC–FT-IR measurement was performed by the use of a combination of a computer controlled *x,y*-stage with a DRIFT unit (diffuse reflectance infrared Fourier transform spectroscopy) and a FT-IR system. The equipment consists of an IFS 48 spectrometer with a narrow band nitrogen cooled MCT detector, a globar as light source and the DRIFT unit, which is constantly purged with dried, carbon dioxide-free air. Commercially available GC-IR Software (OPUS, Bruker) allows the calculation of chromatograms. The special mirror arrangement largely eliminates the specular (Fresnel) reflectance in the 3600 to 1350 $cm^{-1}$ region. The diffuse reflectance, that contains the desired spectral information, is collected and directed to the detector. Library searching was performed with the Bruker software ''search'' in our own HPTLC silica gel library, which consists of more than 400 substances of forensic and pharmaceutical interest. The degree of conformity of the sample and the reference spectra is described by means of a hit quality, wherein the value 1000 resembles maximum fit. For a more detailed description see Ref. [18].

The Gram–Schmidt parameters were: base vectors 20, offset in points 10, size in points 120. The platform advancement was stepwise at 100 μm/s, which enabled co-addition of three interferograms per measuring point at a resolution of 8 $cm^{-1}$. The reference is measured in a similar manner in a substance free zone between two spotted tracks. As function of apodisation the Blackman–Harris 3 Terme has been applied. Demoxepam and aminochlorobenzophenone were separated on silica plates and their DRIFT spectra were added to the library. Three window chromatograms were produced from the integrated total absorption in the region of the strong bands of demoxepam (1720–1680 $cm^{-1}$), nordazepam (1700–1660 $cm^{-1}$) and aminochlorobenzophenone (1640–1600 $cm^{-1}$).

The limit of identification is defined as the lowest amount of substance required for a positive identification that is a hit quality above 600 and first or second place in the list of the library search results. The result ought to be confirmed by fitting the first two reference spectra with the sample spectrum and comparing optically.

### *2.5. Calibration curve and quantitation*

Plates were quantified by linear scanning with a TLC scanner (Densitometer CD 60, Desaga) with a deuterium light source (wavelengh=230 nm) and slit dimensions 4×0.4 mm and a resolution of 8 scans/spot. TLC analysis was performed on 2–14 μl volumes of the standard stock solution. The selection of the working range between 0.2 and 1.4 μg was guided by the loading limit and the required limits of impurities according to the Pharmacopoeia. Validation procedures were carried out according to Funk et al. [19]. As is common in planar chromatography, Mandels test of linearity yielded a significantly better correlation for the second polynomial function.

## 3. Results and discussion

The efficiency of the HPTLC–FT-IR coupling was investigated with regard to the testing of purity using chlordiazepoxide powder (1) which had been stored for a period of about eight years. The monograph of the European Pharmacopoeia for chlordiazepoxide [20] limits the presence of impurities to 0.1% for all unknown substances and 0.05% for aminochlorobenzophenone [2]. The USP monograph [21] for tablets containing chlordiazepoxide tolerates higher values of 4% for demoxepam (3) and 0.1% for aminochlorobenzophenone. The structural formulae are given in Fig. 1.

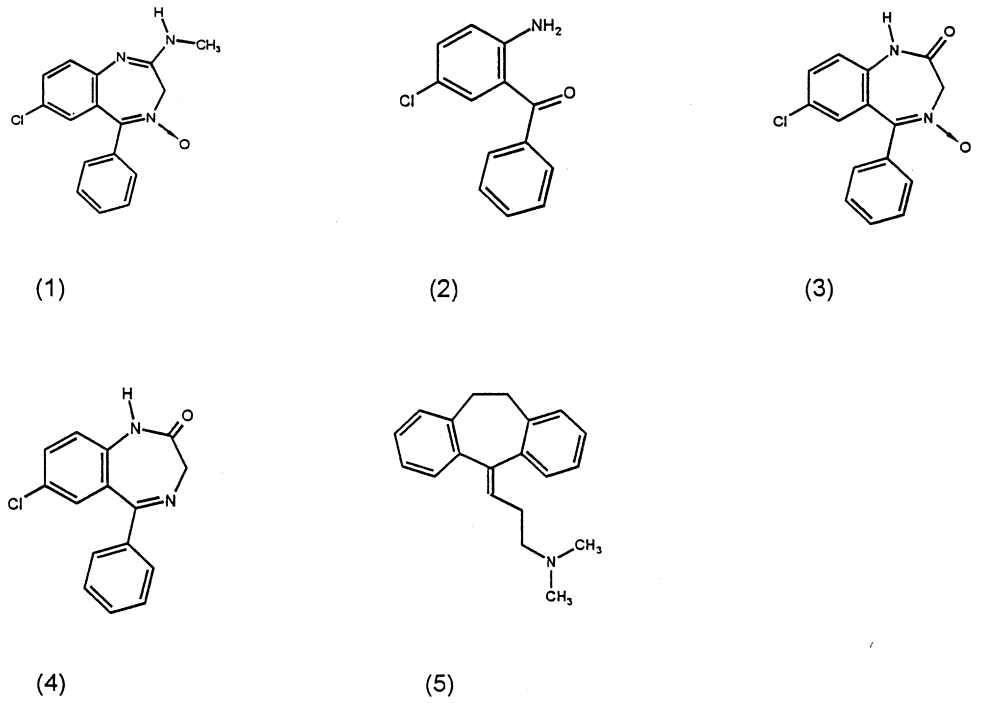


Fig. 1. Chemical structures of chlordiazepoxide, its related compounds and amitriptylin: 1=chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3H-benzodiazepin-4-oxide), 2=2-amino-5-chlorobenzophenone; 3=demoxepam (7-chloro-1,3-dihydro-5-phenyl-2H-benzodiazepin-2-one-4-oxide), 4=nordazepam (7-chloro-1,3-dihydro-5-phenyl-2H-benzodiazepin-2-one), 5=amitriptylin {3-(10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5-yliden)-*N*,*N*-dimethylpropylamin-hydrochloride}.

The TLC development was carried out in accordance with the USP monograph using ethyl acetate as mobile phase, since this is superior to the five-component mixture of the Ph. Eur. The mobile phase in the latter not only takes longer to prepare but yields IR spectra with a greater noise level, likely because water is not completely removed from the plate during the drying process. Another disadvantage are the $R_F$ values leading to partial overlap of amitriptyline and nordazepam. When ethyl acetate is used both chlordiazepoxide and amitriptyline remain in the region of the start, while the impurities have higher $R_F$ values and are completely separated. The TLC analysis of the powder under short-wavelength UV light reveals a further substance peak in addition to chlordiazepoxide at 35 mm ($R_F$=0.58) which is not identical with any of the impurities to be tested according to the USP. In order to identify this substance a Gram–Schmidt chromatogram (Fig. 2) was recorded by scanning the HPTLC plate with an IR beam in a DRIFT unit. By employing the mathematical procedure of vector orthogonalization, the total integral absorption of the sample is measured and yields a universal, substance-nonspecific chromatogram. The peak maximum is used to extract a spectrum directly, whose band position, width and

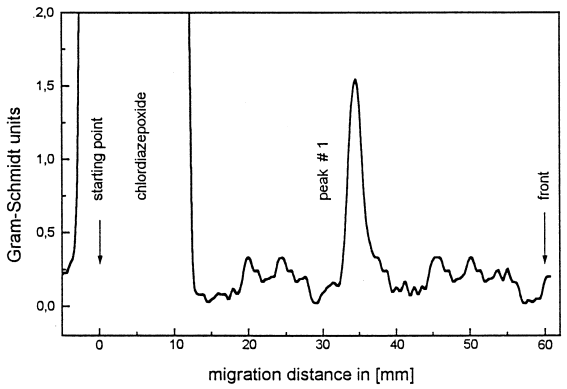


Fig. 2. Gram–Schmidt chromatogram of chlordiazepoxide and of an unknown substance.

intensity are then compared with those of the spectra of the library using the Bruker ''search'' program. The DRIFT spectrum of the unknown compound (Fig. 3) exhibits a strong absorption band (lactam) at 1678 $cm^{-1}$, that indicates formation of a product of hydrolytic degradation. The library search chose nordazepam first on the list with a hit quality of 760 (second: nimetazepam 749).

In order to promote formation of probable decomposition products in the finished medicament and to validate the specificity of the analytical method, powdered tablets (Limbatril tablets, Hoffman-La Roche) were subjected to a stress test. Three products of degradation were detected under the stress test conditions selected: 50°C and 75% relative humidity. The TLC–UV chromatogram reveals the increase in quantity of the impurities as the decomposition progressed (Fig. 4). Quantitation by integration of the IR spectra, the spectra in Kubelka–Munk units and the chromatogram peaks is possible in principle [22]. However, when substances are UV-absorbing, the higher sensitivity and advanced software available for the scanner (automatic zone optimization) make HPTLC–UV coupling preferable for quantitative analysis. Since the large quantity of excess chlordiazepoxide leads to retention and zone enlargement, the determinations of content were performed exclusively from the area units. The

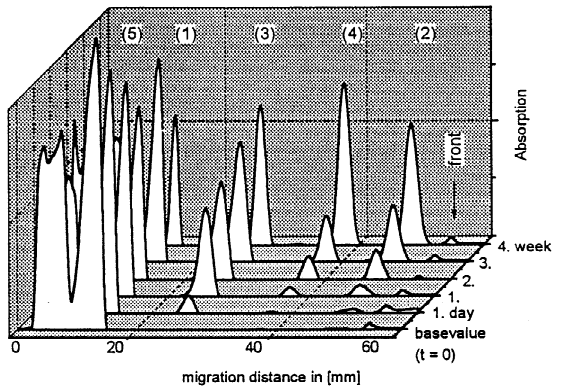


Fig. 4. HPTLC–UV chromatograms of Limbatril tablets and its degradation products.

statistical data are listed in Table 1. They confirm that the method meets all requirements with respect to homogeneity of variance, accuracy and precision. The average from three data points for each sample was taken for quantitative determination. It reveals that the products of decomposition exceeded the permissible limit after only one week (Fig. 5).

As shown before, the identification was achieved by the use of the DRIFT spectra, which were recorded for each sample during the stress test. Only impurities amounting to 0.3% of the whole, depending on the substance, could be detected and iden-

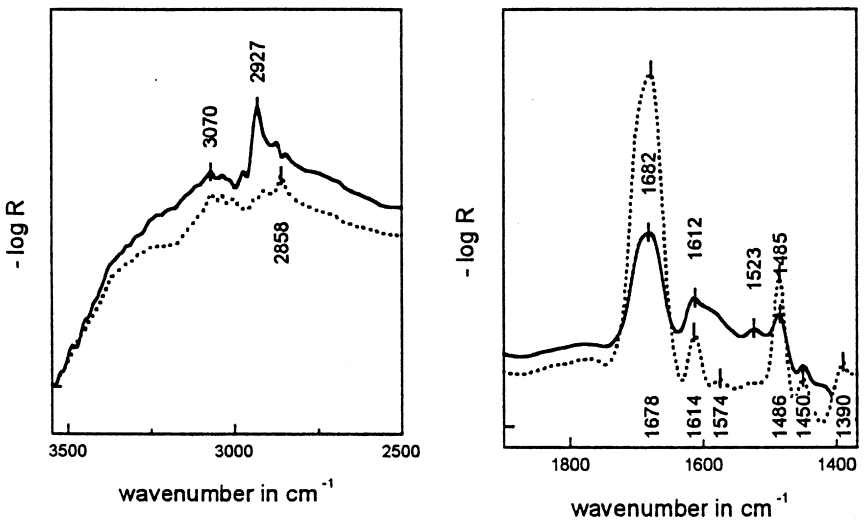


Fig. 3. Comparison of the DRIFT spectra of the sample (dot) and of the nordazepam library reference (line). Hit quality: first place 760 nordazepam (estimated content: 0.4%).

Table 1
Statistical data

| | Demoxepam | Nordazepam | Aminochlorobenzophenone |
|---|---|---|---|
| Calibration curve ($n=7$) | $y=173.9+3945.2x-887.7x^2$ | $y=231.2+4328.8x-1013.6x^2$ | $y=210.2+2963.6x-637.9x^2$ |
| Calibration range (μg) | 0.20–1.41 | 0.21–1.45 | 0.23–1.66 |
| Correlation coefficient ($n=7$) | 0.9997 | 0.9995 | 0.9989 |
| Reproducibility (%)[a] | 3.7 | 3.5 | 3.8 |
| Precision (%)[b] | 4.1 | 1.3 | 4.1 |
| Homogeneity of variance[b] | $P=99\%$ | $P=99\%$ | $P=99\%$ |
| Limit of detection (5 mm band) (μg) | 0.04 | 0.03 | 0.03 |
| Method variation coefficient (%) | 1.1 | 1.5 | 2.2 |

[a] Spiked samples, 15 data points within calibration range.
[b] Spiked samples, 10 data points at bottom and top end of the calibration range, respectively.

tified. The loading capacity of the HPTLC plates is an important, limiting factor for the identification limit. The band-form application procedure allows relatively large volumes to be sprayed on homogeneously in narrow bands. This prevents the sample crystallizing out at the syringe of the linomat and enables good separation. However, the circular IR beam with a diameter of 1.8 mm only covers a part of the 5 mm-substance band. Detection is more sensitive when a substance-specific window chromatogram is recorded. Additionally, a second zone-focusing development at an angle of 90° to the first development direction reduces the identification limit to 0.1–0.05%, which is sufficient for the require-

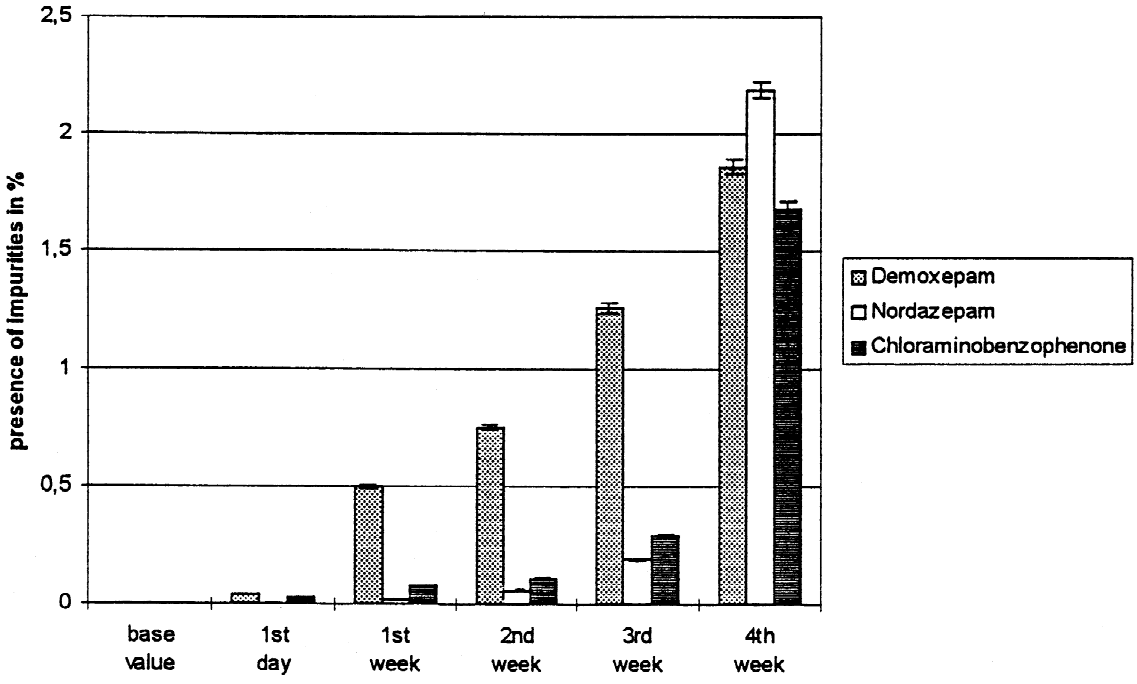


Fig. 5. Quantitative analysis of impurities in Limbatril tablets during stress testing. Identification: hit qualities of the DRIFT spectra. One day: demoxepam (789); first week: demoxepam (737), nordazepam (665), aminochlorobenzophenone (642); second week: demoxepam (743), nordazepam (911), aminochlorobenzophenone (659); third week: demoxepam (876), nordazepam (920), aminochlorobenzophenone (662); fourth week: demoxepam (765), nordazepam (987), aminochlorobenzophenone (666).

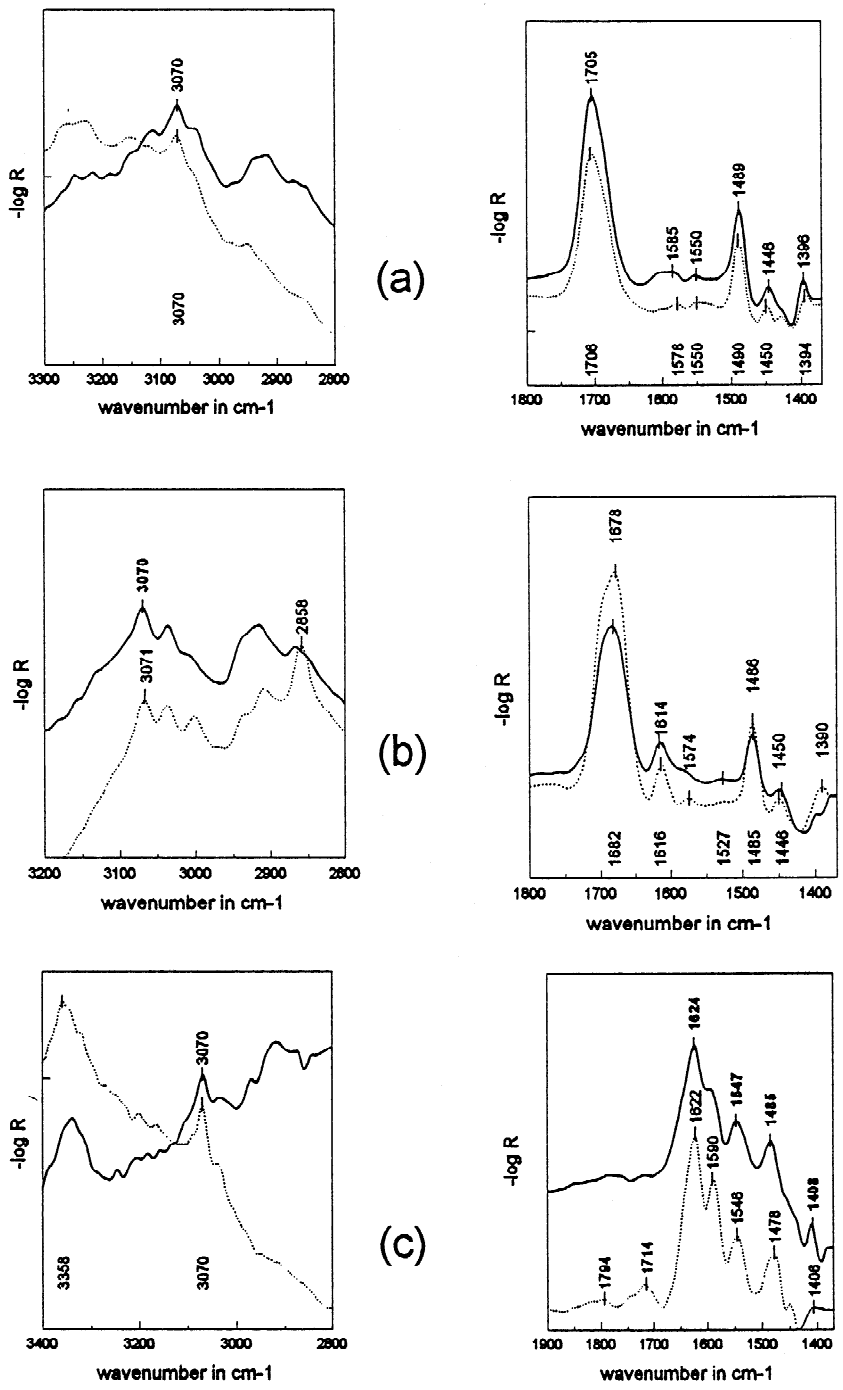


Fig. 6. DRIFT spectra of sample (dot) (a) demoxepam sample: second week 0.84 μg, (b) nordazepam sample: third week 0.99 μg, (c) aminochlorobenzophenone sample: third week 0.76 μg and references (line), respectively.

ments of the monographs. The two-dimensional TLC was employed for all samples containing impurities below 0.3% with the result of unambiguous spectra and positive identification. The recorded DRIFT spectra (see Fig. 6a–c) made it possible to identify the impurities unequivocally as demoxepam (3), nordazepam (4) and aminochlorobenzophenone (2) (for hit qualities see Fig. 5).

This stress test demonstrates that all the occurring degradation products of chlordiazepoxide tablets could be identified with the method proposed. The advantages of the procedure selected are the saving on expensive standard substances together with the high sample throughput as well as the lack of necessity for environmentally damaging spray and dipping reagents. In this example, the Bratton–Mashall colour reaction specified in the monograph for the characterization of the benzophenone as a primary amine can be replaced by the recording of the IR spectra.

## 4. Conclusions

The results demonstrate that direct HPTLC–FT-IR coupling is suitable for the detection and identification of impurities and yields rapid and reliable results. The increased selectivity of IR spectroscopy means that the DRIFT spectra constitute an optimal supplement to the established HPTLC–UV coupling and combine the rapidity and flexibility of TLC with the identification potential of IR spectroscopy. The impurities are identified by comparing the measured spectra with the reference substances of the library. This method gives the opportunity of checking the purity of the product during synthesis or stability tests and identifying the degradation products formed.

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